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A thesis entitled

# **Regulation of E2F in response to DNA damage**

Presented by

**Craig Stevens**

To

**The University of Glasgow**

*For the degree of*

Doctor of Philosophy

**Division of Biochemistry and Molecular Biology**

**Institute of Biomedical and Life Sciences**

**University of Glasgow**

**Scotland**

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To my mum and dad

# Abstract

Transcription factor E2F plays an important role in growth control by co-ordinating early cell cycle events. In addition, certain E2F family members, including E2F-1, are endowed with apoptotic activity. E2F-1 is regulated during cell cycle progression and inducible by cellular stress, such as DNA damage. Within the DNA damage signalling pathway, checkpoint kinases act as effectors of the damage response through phosphorylating key substrates involved in growth control. Here, I report that checkpoint kinase Chk2 regulates E2F-1 activity in response to etoposide. A Chk2 kinase phosphorylation site resides in E2F-1, and undergoes physiological phosphorylation in response to DNA damage. Phosphorylation of E2F-1 by Chk2 leads to protein stabilization, increased half-life and transcriptional activation, and phosphorylated E2F-1 resides in discrete nuclear structures. A dominant-negative derivative of Chk2 blocks the induction of E2F-1, and prevents E2F-1-dependent apoptosis. Moreover, E2F-1 fails to be induced by etoposide in tumour cells that carry mutant *chk2*. Chk2 therefore phosphorylates and activates E2F-1 in the cellular response to DNA damage, and the damage induction of E2F-1 leads to apoptosis. The results suggest a role for E2F-1 in response to stress, perhaps in checkpoint control, and provide a plausible mechanistic and physiological explanation for the tumour suppressor activity of E2F-1.



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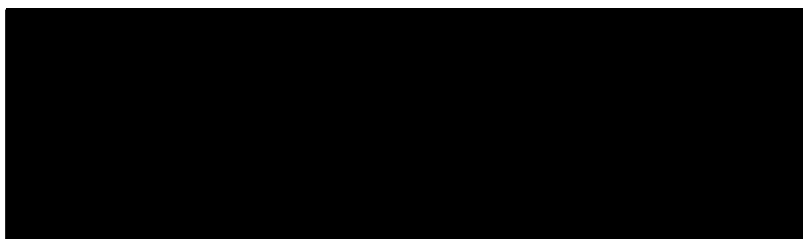
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# Declaration

All the work presented within this thesis was performed entirely by myself and in no way forms part of any other thesis. The work was carried out while I was a graduate student at the Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, UK. I was under the supervision of Professor Nicholas B. LaThangue.



Craig Stevens

September 2003



# **Publications**

The following publications were submitted during the course of the work presented in this thesis.

Stevens, C. and LaThangue, N.B. (2003). E2F and cell cycle control: a double-edged sword. *Arch. Biochem. Biophysics*. **412**, 157-169.

Stevens, C., Smith, L., and LaThangue, N.B. (2003). Chk2 activates E2F-1 in response to DNA damage. *Nat. Cell. Biol.* **5**, 401-409.

Stevens, C. and LaThangue, N.B. (2003). A new role for E2F-1 in checkpoint control. *Cell Cycle*. **2**, 435-437.

# Abbreviations

$\alpha$  – anti-(body)

**Abl** – abelson virus

**Ala (A)** – alanine

**Apaf-1** – Apoptosis protease-activating factor 1

**APL** – acute promyelocytic leukemia

**ARF** – alternative reading frame

**AT** – ataxia telangiectasia

**ATM** – ataxia telangiectasia mutated

**ATP** – adenosine 5'-triphosphate

**ATR** – ataxia telangiectasia and Rad3-related

**$\beta$ -gal** –  $\beta$ -galactosidase

**Bad** – Bcl-2 antagonist of cell death

**Bak** – Bcl-2 antagonist/killer

**BASC** – BRCA1-associated genome surveillance complex

**Bax** – Bcl-2 associated protein X

**Bcl-2** – B-cell lymphoma-2

**Bid-1** – BH-3 interacting domain death antagonist 1

**Bok** – Bcl-2-related ovarian killer

**BRCA1** – breast cancer gene 1

**BRCT** – BRCA1 C-terminus

**BRG1** – human brahma related gene 1

**C/EBP $\alpha$**  – CCAAT/enhancer-binding protein  $\alpha$

**Cdc** – cell division cycle

**cdi** – cdk inhibitor

**cdk** – cyclin dependent kinase

**Chip** – chromatin immunoprecipitation

**Chk** – checkpoint kinase

**DAPI** – 4, 6-diamino-2-phenylindole

**DHFR** – dihydrofolate reductase

**DMEM** – Dulbecco's modification of eagles medium

**DMSO** – dimethylsulphoxide

**DNA** – deoxyribonucleic acid

**DNA-PK** – double-stranded DNA-activated protein kinase

**DP** – DRTF1-protein

**DRTF1** – differentiation regulated transcription factor 1

**d/s** – double-stranded

**DTT** – dithiothreitol

**EC** – embryonal carcinoma

**ECL** – enhanced chemiluminescence

**EDTA** – ethylene diamine tetra-acetic acid

**ES** – embryonic stem cells

**E1A** – adenovirus early region 1A

**E2F** – E2A binding factor

**FACS** – fluorescence activated cell sorting

**FCS** – foetal calf serum

**FHA** – forkhead-associated domain

**GST** – glutathione-S-transferase

**G0** – quiescence

**G1** – gap phase 1

**G2** – gap phase 2

**h** – hour

**HA** – hemagglutinin protein

**HAT** – histone acetyltransferase

**HBRM** – human brahma

**HBS** – HEPES buffered saline

**HDAC** – histone deacetylase

**HEPES** – N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]

**His** – 6x histidine tag

**HPV** – human papilloma virus

**HP1** – heterochromatin protein 1

**IB** – immunoblot

**Ink4a** – inhibitor of cdk4

**IP** – immunoprecipitation

**IPTG** – isopropyl- $\beta$ -D-thiogalactopyranoside

**IR** – ionising radiation

**IVT** – *in vitro* transcribed and translated

**K** – lysine

**kDa** – kilodalton

**LFS** – Li-Fraumeni syndrome

**Luc** – luciferase

**M** – molar

**MDC1** – mediator of damage checkpoint protein 1

**mg** – milligram

**µg** – microgram

**min** – minute(s)

**ml** – millilitre

**µl** – microlitre

**mM** – millimolar

**µM** – micromolar

**MCM** – minichromosome maintenance

**MDM2** – murine double minute 2

**MEF** – mouse embryonic fibroblast

**Mre11** – Mre11, Rad50 and Nbs1 complex

**mRNA** – messenger RNA

**MTase** – histone methyltransferase

**Myc** – myelocytomatosis viral oncogene

**NBS** – Nijmegen breakage syndrome

**NES** – nuclear export signal

**NFBD1** – nuclear factor with BRCT domains protein 1

**NFκB** – nuclear factor of immunoglobulin k locus in B cells

**NHEJ** – non-homologous end-joining pathway

**NLS** – nuclear localisation signal

**NP-40** – nonidet P40

**ORC1** – origin recognition complex subunit 1

**P** – phosphate group

**PAGE** – polyacrylamide gel electrophoresis

**PBS** – phosphate buffered saline

**PCAF** – p300/CBP associated factor

**PcG** – polycomb group proteins

**PCNA** – proliferating cell nuclear antigen

**Phe** – phenylalanine

**PI-3** – phosphatidylinositol-3-kinase

**PIKK** – phosphatidylinositol-3-kinase-like kinase

**PML** – promyelocytic leukemia protein

**PMSF** – phenylmethanesulfonyl fluoride

**PPAR $\gamma$**  – peroxisome proliferator-activated receptor  $\gamma$

**pRb** – retinoblastoma tumour suppressor protein

**p300/CBP** – p300/CRE binding protein

**RDS** – radioresistant DNA synthesis

**RNA** – ribonucleic acid

**RYBP** – ring 1 and YY1-binding protein

**SCD** – SQ/TQ cluster domain

**SCF** – skip, cullen, F-box

**SDS** – sodium dodecyl sulphate

**Ser (S)** – serine

***Sf9*** – *spodoptera frugiperda*

**SiRNA** – small interfering RNA

**Smad** – *C.elegans* SMA and *Drosophila* MAD

**SV40** – simian virus 40

**SWI2/SNF2** – switching defective/sucrose nonfermenter

**TBE** – tris-borate EDTA

**TBP** – TATA binding protein

**TCR** – T cell receptor

**TCR-AICD** – TCR activation-induced cell death

**TGF $\beta$**  – transforming growth factor  $\beta$

**Thr (T)** – threonine

**Tlk** – tousel like kinases

**TNF** – tumour necrosis factor

**TNFR** – tumour necrosis factor receptor

**TopBP1** – DNA topoisomerase II $\beta$  binding protein 1

**Tris** – tris(hydroxymethyl)methylamine

**Trp** – tryptophan

**Tween 20** – polyoxyethylene sorbitan monolaurate

**w/v** – weight per volume

**v/v** – volume per volume

**(+/+)** – wild-type

**(+/-)** – heterozygous mutant

**(-/-)** – homozygous mutant

**53BP1** – p53 binding protein 1

# Chapter 1

## Introduction

### 1.1 The mammalian cell cycle

The mammalian cell cycle is divided into four distinct phases, referred to as G1, S, G2 and M-phase. The two gap periods, G1 and G2, are growth phases that precede DNA synthesis and replication (S-phase), and cell division or mitosis (M-phase). In late G1-phase and prior to entering S-phase, cells pass through the restriction point. The restriction point is the point in the mammalian cell cycle when cells become committed to entering S-phase and completing the cell cycle, independently of further growth factor signalling (Sherr, 1996).

Passage through the restriction point and entry into S-phase is coordinated by the sequential activation and inactivation of the G1 cyclin-dependent kinases (cdks). Cdk activity is regulated by the temporal synthesis and binding of specific regulatory subunits, called cyclins, the association and dissociation of cdk inhibitors (cdi), as well as inhibitory and activating phosphorylation events (Sherr, 1996). Mitogenic growth factors induce genes encoding D-type cyclins, and the cyclins assemble with their catalytic partners to form active cdk 4/6-cyclinD complexes. Active cdks subsequently phosphorylate key growth regulating proteins, such as the retinoblastoma tumour suppressor protein (pRb), which thereafter allows cells to traverse the G1 phase. The subsequent activation of other genes required for cell cycle



progression including cyclinE (which forms an active complex with cdk2 and facilitates progressive pRb phosphorylation), and enzymes involved in DNA replication, is sufficient to commit cells to S-phase. The activity of pRb is intimately connected with the restriction point, and once cells pass the restriction point the cell cycle is self-regulated, unless DNA becomes damaged or defects are encountered during mitosis, which activates checkpoints and delays the cell cycle.

## **1.2 History of E2F**

Historically, E2F was referred to as DRTF1/E2F, reflecting the early studies which elucidated its activity (Nevins, 1992). E2F was defined as a cellular factor required for the adenovirus early region 1A (E1A) transforming protein to mediate the transcriptional activation of the viral E2A promoter (Nevins, 1992). On the other hand, DRTF1 (differentiation-regulated transcription factor 1) was identified as a transcription activity whose DNA binding activity decreased during the differentiation of F9 embryonal carcinoma (EC) stem cells (La Thangue and Rigby, 1987). The consensus DNA binding site for DRTF1 and E2F subsequently were found to be similar which, together with other observations, suggested that DRTF1 and E2F were related. In this thesis the combined activity will be referred to as E2F.

The E2F consensus binding site is “TTTCGCGC”, present in the adenovirus E2A promoter (Nevins, 1992), and predominantly within the promoters of cellular genes required for cell division and apoptosis (Dyson, 1998). Identified E2F target genes include cell cycle regulators such as the

cyclins E, A and D1, Cdc2, and Cdc25A, enzymes involved in DNA synthesis like dihydrofolate reductase (DHFR), DNA polymerase  $\alpha$  and thymidine kinase, and proteins essential for DNA replication including Cdc6, ORC1 and the minichromosome maintenance (MCM) proteins (Dyson, 1998; Table 1.1). Apoptotic E2F target genes include apoptosis protease-activating factor 1 (Apaf-1), p73 and ARF (Moroni *et al.*, 2001; Irwin *et al.*, 2000; Lissey *et al.*, 2000; Bates *et al.*, 1998).

### **1.3 The retinoblastoma family of proteins**

Interest in E2F increased considerably when, through studies on the cellular targets of viral oncoproteins, E2F was identified to be important for the function of pRb (Bandara and La Thangue, 1991; Chellappan *et al.*, 1991). The Rb gene possesses the properties of a classical tumour suppressor gene, being absent or mutated in at least one third of all human tumours (Weinberg, 1995; Sherr, 1996). Single allelic mutations in *Rb* predispose sufferers to a wide variety of tumour types (Weinberg, 1995), and familial inactivating mutations in *Rb* are associated with the rare paediatric eye tumour retinoblastoma (Friend *et al.*, 1986).

pRb is the prototype member of a family of proteins, referred to as the pocket proteins, that includes p107 and p130 (Wang *et al.*, 1997). pRb shares significant homology with p107 and p130, particularly in the “pocket region”. The large pocket region of pRb (amino acids 395-876) binds to E2F, and the small pocket region (amino acids 379-792) binds to the LXCXE peptide (Wang *et al.*, 1997). Viral oncoproteins, including adenovirus E1A, SV40 large T

Table 1.1 E2F regulated genes

(Adapted from Helin, 1998 and data also from Muller *et al.*, 2001; Ren *et al.*, 2002; Ma *et al.*, 2002; Stanelle *et al.*, 2002; Polager *et al.*, 2002).

DNA synthesis	Proliferation	Apoptosis	DNA repair
DNA polymerase $\alpha$ thymidine kinase DHFR ORC1 Cdc6 MCM PCNA	CyclinD1 cyclinE cyclinA cdc2 cdk2 p107 pRb c-myc E2F-1,2,3,4 & 5 DP-1 Cdc25A Cdc25C	P19 <sup>ARF</sup> p73 Apaf-1 Caspase 3 Caspase 7 Bok Bad Bak Bid-1	MSH2 MSH6 RPA2 RPA3 BRCA1 PCNA

antigen, HPV E7 and some cellular proteins, such as histone deacetylases (HDACs) and cyclinD1, possess an LXCXE motif in their protein sequence that directs their association with pRb (Mulligan and Jacks, 1998; Figure 1.1). The ability of viral oncoproteins to transform cells is in part dependent on overcoming the growth suppressive activities of pRb. They achieve this by displacing cellular proteins that interact with the pocket, thereby leading to loss of pocket protein function and normal growth control (Bandara and La Thangue, 1991; Nevins, 1992). Despite the sequence similarity between pRb, p107 and p130, *Rb* is the only gene of the family that is frequently mutated in tumour cells (Weinberg, 1995).

To study the physiological role of each pocket protein, mice lacking *Rb*, *p107* and *p130* have been developed. Mice deficient in *Rb* exhibit embryonic lethality, dying between embryonic day E13.5 and E15.5, and suffer from abnormal differentiation in a variety of tissues, most probably due to inappropriate cell cycle entry and elevated apoptosis (Clarke *et al.*, 1992). The analysis of *p107*<sup>-/-</sup> and *p130*<sup>-/-</sup> mice supports the idea that p107 and p130 act in a distinct manner from pRb. Thus, in contrast to *Rb*<sup>-/-</sup>, *p107*<sup>-/-</sup> and *p130*<sup>-/-</sup> mice survive to term and show no increase in the incidence of tumour formation. However, *p107*<sup>-/-</sup>/*p130*<sup>-/-</sup> double knock-out mice exhibit embryonic lethality suggesting functional redundancy between p107 and p130 (Cobrinik *et al.*, 1996). It has also been observed that deficiency of p107 enhances the developmental abnormalities of *Rb*<sup>+/-</sup> embryos, arguing for some degree of functional redundancy between p107 and pRb (Lee *et al.*, 1996). These observations, together with the lack of p107 and p130 mutations in human tumours, indicate that p107 and p130 are unlikely to act as tumour suppressors.

## 1.4 Control of pRb activity

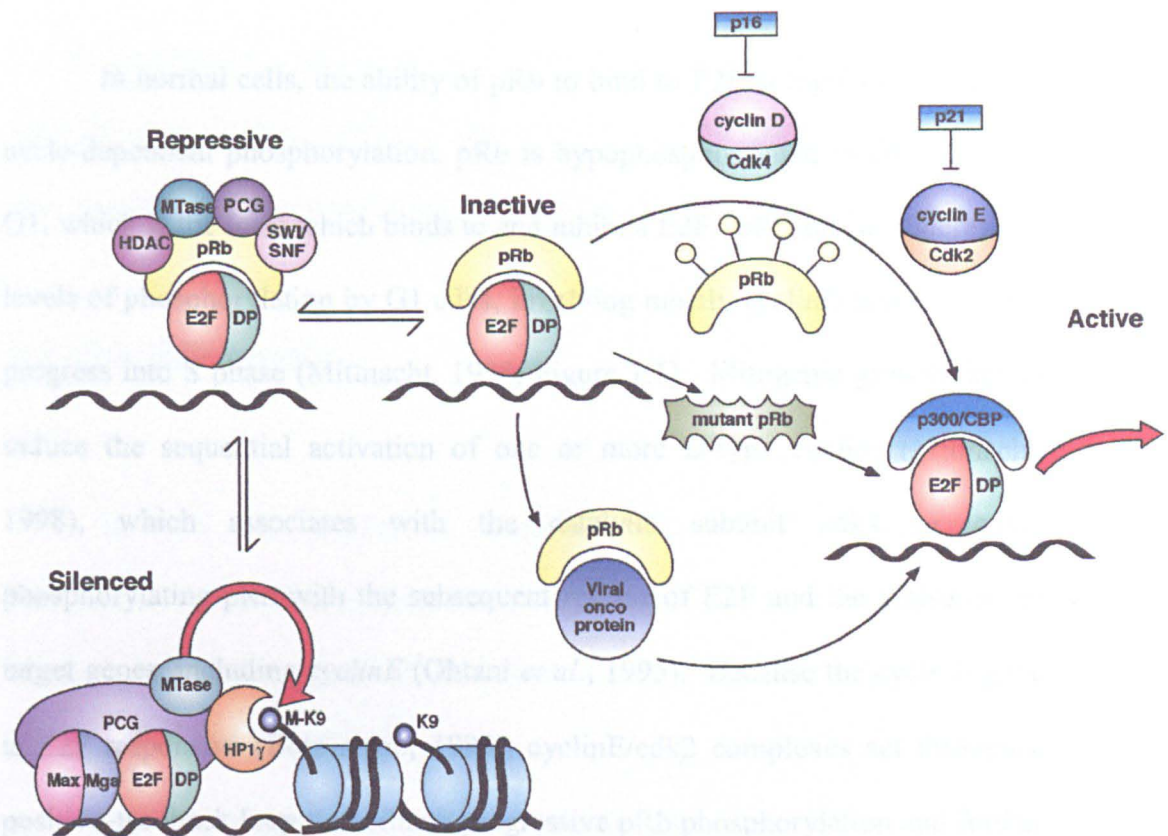


Figure 1.1 Regulation of E2F activity.

In non-proliferating cells, the E2F/DP heterodimer binds pocket proteins such as pRb. During early cell cycle progression G1 cyclin-dependent kinase complexes (cyclinD/Cdk4 and cyclinE/Cdk2) phosphorylate pRb, releasing E2F. E2F becomes transcriptionally active through interaction with transcription co-factors such as p300/CBP. In tumour cells, mutant pRb fails to bind E2F. Similarly, viral oncoproteins bind pRb to hinder its interaction with E2F. In addition, p16 can be inactivated, or cyclinD1 over-expressed, in cancer cells. The pRb/E2F complex becomes an active transcriptional repressor by recruiting proteins, such as HDAC, Mtase, PCG and SW1/SNF, that alter the chromatin environment to favour transcriptional inactivity.

In quiescent G0 cells, E2F heterodimers such as E2F-6 may nucleate the assembly of another type of chromatin remodelling complex containing Mtase, PCG, and HP1, and involve other sequence specific transcription factors like Max and its partner Mga. By modifying the chromatin environment of target genes this complex co-ordinates long term silencing of cell cycle regulated genes. (Figure from Stevens and LaThangue, 2003).

## 1.4 Control of pRb activity

In normal cells, the ability of pRb to bind to E2F is regulated by its cell cycle-dependent phosphorylation. pRb is hypophosphorylated in G0 and early G1, which is the form which binds to and inhibits E2F. pRb sustains increasing levels of phosphorylation by G1 cdks, involving mostly cyclinD and E, as cells progress into S phase (Mittnacht, 1998; Figure 1.1). Mitogenic growth factors induce the sequential activation of one or more D-type cyclins (Mittnacht, 1998), which associates with the catalytic subunit cdk4 or cdk6, phosphorylating pRb with the subsequent release of E2F and the activation of target genes, including *cyclinE* (Ohtani *et al.*, 1995). Because the cyclinE gene is E2F responsive (Botz *et al.*, 1996), cyclinE/cdk2 complexes act through a positive-feedback loop to facilitate progressive pRb phosphorylation and further release of E2F (Lundberg *et al.*, 1998), producing a rapid rise of cyclinE/cdk2 required to allow cells to initiate DNA replication (Sherr, 1996).

The kinase activity of cyclin/cdk complexes in turn is negatively regulated by cdks, which belong to one of two families based on their structural relationship (Sherr and Roberts, 1999). The first family includes the Ink4a proteins (inhibitors of cdk4), which specifically inhibit the catalytic subunits cdk4 and cdk6. Four such proteins, namely p16<sup>INK4a</sup>, p15<sup>INK4b</sup>, p18<sup>INK4c</sup> and p19<sup>INK4d</sup>, have been described. The second Cip/Kip family act more generally and affect the activities of cyclin D, E and A-dependent kinases, and include p21<sup>Cip1</sup>, p27<sup>Kip1</sup> and p57<sup>Kip2</sup>, which bind to both the cyclin and cdk subunit (Sherr and Roberts, 1999). Under conditions of cellular stress, such as DNA damage, replication inhibition, or in response to inhibitory signals like

transforming growth factor  $\beta$  (TGF $\beta$ ), cell cycle progression is stalled through the action of cdis, leading to the inhibition of one or more cyclin/cdk complexes and the maintenance of pRb in the hypo-phosphorylated growth suppressing state (Sherr and Roberts, 1999; Figure 1.1). Thus, pRb integrates both positive and negative growth regulating signals with the transcription machinery.

## 1.5 The E2F family

In cells E2F activity is comprised of a family of heterodimeric transcription factors where each heterodimer consists of one member of the E2F family bound to a member of the DP family (Dyson, 1998; Table 1.2). In mammalian cells, six E2F family members have been identified (E2F-1 to E2F-6), whilst two members of the DP family, DP-1 and DP-2, have been characterised. All possible combinations of E2F/DP complexes can exist *in vitro*, potentially allowing for the formation of an array of E2F complexes in cells (Dyson, 1998). This complexity may be even greater if mechanisms such as alternative splicing, which occurs in DP-2 RNA (de la Luna *et al.*, 1996), or for E2F-3 the expression of a second transcript derived from an additional start site within the locus (Adams *et al.*, 2000), contribute to E2F complexes. *In vitro* individual E2F/DP complexes recognise similar nucleotide sequences (Lees *et al.*, 1993; Buck *et al.*, 1995; Zhang and Chellappan, 1995), although some evidence suggests that different heterodimer complexes preferentially bind to specific E2F sequences (Tao *et al.*, 1997). It is not yet clear whether E2F complexes target preferred E2F-regulated promoters in cells.

Table 1.2 Summary of the properties of E2F and DP family members

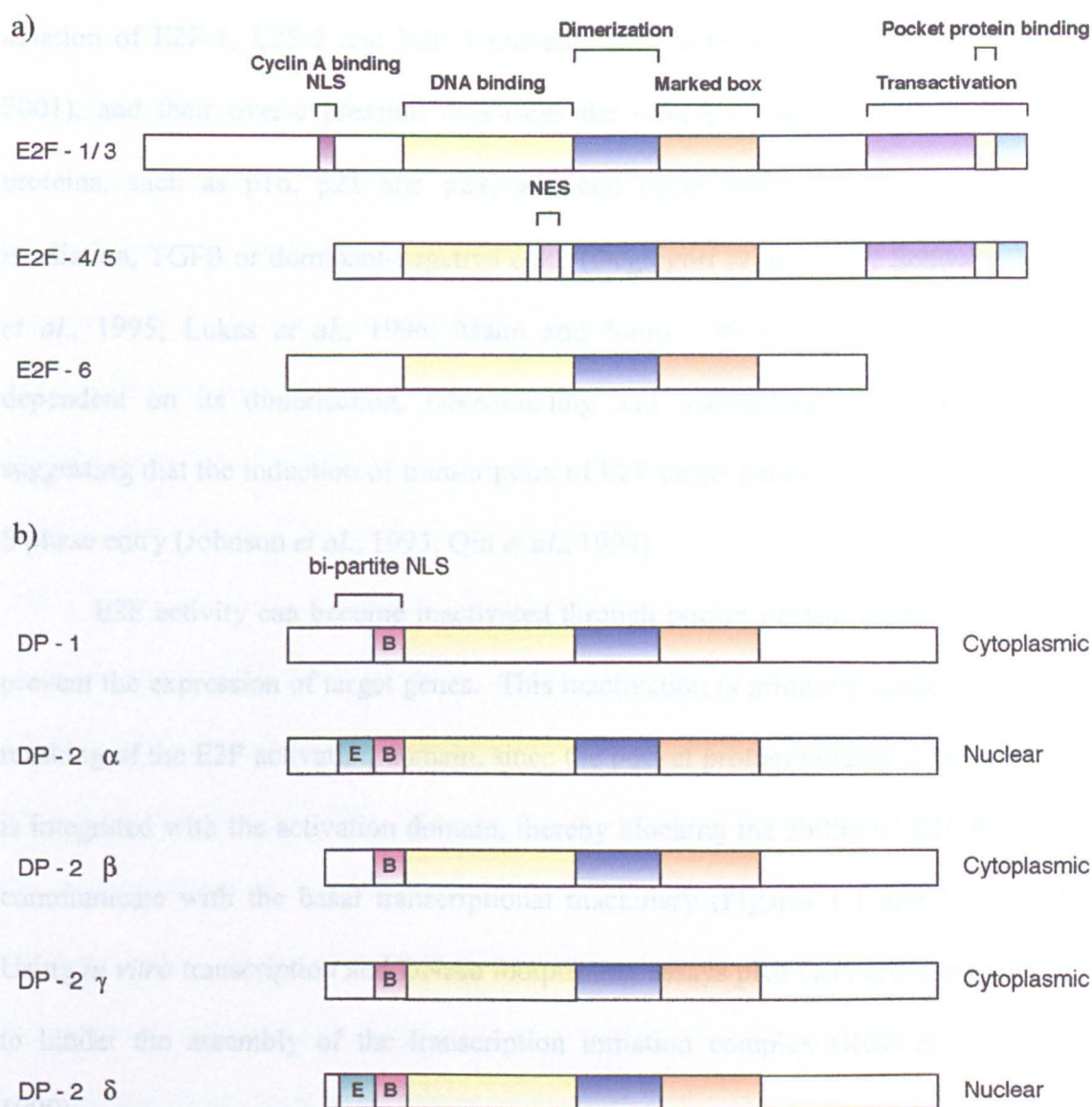
Protein	Expression	Pocket protein	Cell cycle phase complexed with pocket protein	Nuclear localisation signal	S phase induction	Induction of apoptosis	Tumour suppressor activity	Knock-out mouse		Direct interaction with repressive chromatin modifying enzymes	CyclinA/cdk2
								Viable	Phenotype		
E2F-1	G1/S	pRb	G0, S, G <sub>1</sub>	Yes	+++	+++	Yes	Yes	tumours	No	Yes
E2F-2	G1/S	pRb	G0, S, G <sub>1</sub>	Yes	+++	+	No	Yes	immunologic defects	No	Yes
E2F-3	G1/S	pRb	G0, S, G <sub>1</sub>	Yes	+++	+	No	Yes	reduced gene expression	No	Yes
E2F-4	Constitutive	p107, p130	S, G0	No	+	+	No	No	developmental defects	No	No
E2F-5	Constitutive	p130, p107	G0, S	No	+	+	No	No	hydrocephaly	No	No
E2F-6	Constitutive	-	-	Yes	-	-	No	-	-	Yes	No
DP-1	Constitutive	pRb, p107, 130	G0, S, G <sub>1</sub>	No	+	-	No	-	-	No	No
DP-2	Constitutive	pRb, p107, p130	G0, S, G <sub>1</sub>	Yes, dependent on mRNA splicing	+	-	-	-	-	No	No



The E2F family can be further divided into three sub-groups based on sequence homology; E2F-1, E2F-2 and E2F-3 represent one group, and E2F-4 and E2F-5 a second (Figure 1.2). Functional similarities are evident within each group; E2F-1, E2F-2 and E2F-3 share an N-terminal cyclinA/cdk binding domain and a canonical basic nuclear localisation signal (NLS), both of which are absent in E2F-4 and E2F-5, which instead possess a nuclear export signal (NES; Gaubatz *et al.*, 2001). Moreover, E2F/DP heterodimers interact with pocket proteins with a specificity that is largely determined by the E2F component (Dyson, 1998 and Table 1.2); E2F-1, E2F-2 and E2F-3 preferentially associate with pRb, whereas E2F-4 and E2F-5 predominantly interact with p107 and p130 (Dyson, 1998). E2F-6 represents the third sub-group, and acts principally as a transcriptional repressor through a distinct pocket protein-independent manner (Morkel *et al.*, 1997). In fact, E2F-6 diverges considerably from the other E2F family members, sharing almost no homology outside the core DNA binding and dimerisation domains, and possesses a truncated C- and N-terminal region relative to the other E2F sub-groups (Morkel *et al.*, 1997 and Figure 1.2).

## **1.6 E2F-1, E2F-2 and E2F-3**

E2F-1, E2F-2 and E2F-3 activate E2F responsive genes and drive cellular proliferation (DeGregori *et al.*, 1997). Over-expression of each protein is sufficient to induce quiescent cells to re-enter the cell cycle (Johnson *et al.*, 1993; Asano *et al.*, 1996; Lukas *et al.*, 1996; Wang *et al.*, 1998), and dominant-negative mutants block S phase entry (Wu *et al.*, 1996). Further, the combined



**Figure 1.2 Structural comparison of E2F and DP family members.**

a) The E2F family can be divided into three subgroups based on sequence homology. E2F-1, E2F-2 and E2F-3 represent one group, E2F-4 and E2F-5 a second and E2F-6 represents a third subgroup. The E2F-1 subgroup have an N-terminal cyclin/cdk binding site and a nuclear localisation signal (NLS). E2F-4 and E2F-5 have truncated N-terminal regions and lack cyclin/cdk binding site and an NLS, instead possessing a nuclear export signal (NES). The positions of pocket protein binding and *transactivation* domains are indicated.

b) Summary of DP-1 and DP-2 family members. E2F and DP proteins share a conserved DNA binding and dimerisation domain. Four alternatively-spliced forms of DP-2 have been described which either are cytoplasmic ( $\beta$  and  $\gamma$ ) or nuclear ( $\alpha$  and  $\delta$ ). The expression of specific DP proteins may influence E2F localisation. (Figure from Stevens and LaThangue, 2003).

ablation of E2F-1, E2F-2 and E2F-3 prevents entry into S phase (Wu *et al.*, 2001), and their over-expression over-rides the effects of growth inhibitory proteins, such as p16, p21 and p27, and cell cycle arrest induced by  $\gamma$ -irradiation, TGF $\beta$  or dominant-negative cdk2 (Degregori *et al.*, 1995; Schwarz *et al.*, 1995; Lukas *et al.*, 1996; Mann and Jones, 1996). This ability is dependent on its dimerisation, DNA-binding and *transactivation* domains, suggesting that the induction of transcription of E2F target genes is required for S phase entry (Johnson *et al.*, 1993; Qin *et al.*, 1994)

E2F activity can become inactivated through pocket protein binding, to prevent the expression of target genes. This inactivation is primarily caused by masking of the E2F activation domain, since the pocket protein binding domain is integrated with the activation domain, thereby blocking the ability of E2F to communicate with the basal transcriptional machinery (Figures 1.1 and 1.2). Using *in vitro* transcription and DNase footprinting assays pRb has been shown to hinder the assembly of the transcription initiation complex (Ross *et al.*, 1999).

In addition, pRb is able to mediate active repression of E2F targets through nucleating the assembly of a dominantly-acting repressor complex (Zhang *et al.*, 1999, Ross *et al.*, 2001; Figure 1.1). Once targeted to the promoter by E2F, pRb recruits proteins endowed with chromatin modifying activity, including HDACs, the histone methyltransferase (MTase) SUV39H1, human brahma (HBRM) and human brahma-related gene 1 (BRG1) - human homologues of the yeast SWI2/SNF2 proteins that possess nucleosome-remodelling activities (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998; Nielsen *et al.*, 2001; Zhang *et al.*, 2000).

The HDACs are a family of proteins, of which HDAC 1, 2 and 3 have been reported to interact with pRb (Harbour and Dean, 2000). HDAC removes the acetyl groups from lysine (K) residues within the tail regions of histones which appears to facilitate the condensation of nucleosomes into inactive chromatin (Harbour and Dean, 2000). Lysine residues that are hypo-acetylated through HDACs may subsequently become methylated by MTases. Specifically MTases such as SUV39H1 methylate K9 in the tail region of histone H3, to create a high affinity binding site for members of the heterochromatin protein 1 (HP1) family of repressor proteins, which recognise methylated lysine residues (Lachner *et al.*, 2001; Bannister *et al.*, 2001). HP1 proteins localise to regions of heterochromatin and are believed to induce long term transcriptional silencing (Lachner *et al.*, 2001; Bannister *et al.*, 2001). The interplay amongst the post-translational modifications of histone tails, which also includes phosphorylation, provides the basis of the “histone code”, which proposes that the pattern of modifications within the histone tail dictate whether chromatin is in an open or closed state (Jenuwein and Allis, 2001).

Both BRG1 and HBRM are two ATPase components of the human SWI/SNF chromatin-remodelling complex. These proteins influence the access of transcription factors to promoters by altering nucleosomal structure and nucleosome position in a manner dependent on ATP hydrolysis (Harbour and Dean, 2000). Interestingly, recent results indicate that pRb can recruit HDAC and SWI/SNF either separately or together (Zhang *et al.*, 2000). The pRb/SWI/SNF complex is sufficient to repress cyclinA and cdc2 genes, whereas repression of cyclinE and E2F-1 genes also requires HDAC (Zhang *et al.*,

2000). It appears possible that distinct types of repressor complex regulate different sets of genes.

## **1.7 E2F-4 and E2F-5**

E2F-4 and E2F-5 were cloned by virtue of their association with pocket proteins or DP-1 (Beijersbergen *et al.*, 1994; Buck *et al.*, 1995; Hijmans *et al.*, 1995). Their sequence and organisation differs markedly from E2F-1, E2F-2 and E2F-3 (Figure 1.2), which reflects their unique modes of regulation. Whereas E2F-1, -2 and -3 are under cell cycle control, with levels peaking as cells approach the G1/S phase boundary, E2F-4 and E2F-5 are uniformly expressed with significant levels detectable in quiescent (G0) cells (Dyson, 1998).

Endogenous E2F-4 and E2F-5 complexes can act as repressors of E2F-responsive genes, which reflects in part their regulation through sub-cellular localisation (de la Luna *et al.*, 1996; Allen *et al.*, 1997; Muller *et al.*, 1997; Verona *et al.*, 1997). Thus, E2F-4 and -5 are predominately cytoplasmic, and need to assemble with a pocket protein to be transported into nuclei (Allen *et al.*, 1997; Verona *et al.*, 1997). At a mechanistic level, this means that E2F-4 and -5 bind to target genes in a transcriptionally inactive state. Nevertheless, and in a similar fashion to E2F-1, both E2F-4 and -5 possess an intrinsic transcriptional activation domain which becomes active once pocket proteins are released (Dyson, 1998). In addition, E2F-4 and -5 can undergo nuclear import by association with DP-2 which, in contrast to DP-1, possesses an intrinsic NLS (de la Luna *et al.*, 1996; Lindeman *et al.*, 1997; Magae *et al.*,

1996), suggesting that the expression of specific DP proteins can influence the overall spectrum of nuclear E2F activity (de La Luna *et al.*, 1996).

The observation that E2F-4 and -5 pocket protein complexes predominate in G0/G1 cells suggests that they are primarily involved in repression during early cell cycle progression (Morberg *et al.*, 1996). In this respect, mutation of the E2F binding sites in some E2F-responsive promoters (such as *B-myb*, *cdc2*, and *E2F-1*) leads to increased expression during G0/G1, consistent with transcriptional repression through the E2F site (Dalton, 1992; Lam *et al.*, 1993; Hsiao *et al.*, 1994). *In vivo* significant levels of the E2F-4/p130 complex occupy E2F sites during G0/G1, with low levels of local histone acetylation (Takahashi *et al.*, 2000). Reduced occupancy by E2F-4 occurs in late G1 phase when gene expression is induced, correlating with the appearance of E2F-1, E2F-2 and E2F-3, and an increase in acetylation of histones H3 and H4 (Takahashi *et al.*, 2000). Significantly, the decrease in binding of E2F-4 to E2F sites correlates with the dissociation of E2F-4/DP-pocket protein complexes, together with re-localisation of E2F-4/DP to the cytoplasm (Takahashi *et al.*, 2000). By genomic footprinting the E2F sites of the *B-myb*, *cdc2* and *cyclinA* promoters are occupied in G0 and early G1 phase cells, when the E2Fs are likely to act in repressing transcription as part of larger pocket protein complexes (Tommasi *et al.*, 1995; Zwicker *et al.*, 1996; Huet *et al.*, 1996).

Interestingly, E2F-5 can be phosphorylated on a single threonine residue within its activation domain by cyclinE/cdk2 which augments the recruitment of p300/CRE binding protein (p300/CBP) family of co-activators, stimulating activation of E2F responsive genes in late G1 (Morris *et al.*, 2000). This study

implies a positive auto-regulatory mechanism for E2F-dependent transcription, and supports the notion that E2F-4/-5 family members play a role in the activation of target genes.

Another level of complexity is suggested by a recent report (Chen *et al.*, 2002) which described how E2F-4/-5 and p107 can act as transducers of the inhibitory growth factor TGF $\beta$ , independently of cdis. The *C.elegans* SMA and *Drosophila* MAD (Smad3) protein mediates transcriptional activation of its target genes, which include cdis, in response to TGF $\beta$  treatment. Smad3 can also mediate transcriptional repression of the growth promoting gene *c-myc*. A Smad3, E2F-4/-5, DP-1, p107 complex pre-exists in the cytoplasm and is translocated into the nucleus in response to TGF $\beta$ , where it associates with Smad4. Smad4 allows the complex to recognise a composite Smad-E2F binding site in the *c-myc* promoter leading to repression. In this context at least, E2F-4/-5 and p107 act in a Smad3 protein complex as transducers of TGF $\beta$  receptor signals upstream of cyclin-dependent kinases.

## 1.8 E2F-6

E2F-6 differs from the other E2Fs; whilst its DNA-binding and dimerisation domains are conserved, it lacks the C-terminal *transactivation* and pocket-protein binding domains (Morkel *et al.*, 1997; Figure 1.2). This has led to the suggestion that E2F-6 may act as a repressor of E2F-responsive genes by binding to promoters, preventing activation by E2F family members. However, recent studies revealed that E2F-6 recruits multiple cellular factors to form a potent repressive complex, which it targets to E2F-responsive promoters

(Trimarchi *et al.*, 2001; Ogawa *et al.*, 2002). These factors include Ring1 and YY1-binding protein (RYBP), members of the polycomb group (PcG) of proteins, which are involved in maintaining transcriptional repression. PcG proteins bind to core promoter regions and general transcription factors *in vitro*, and depletion of PcG proteins leads to the de-repression of developmentally regulated genes (Breiling *et al.*, 2001). In addition, E2F-6 recruits chromatin modifying proteins to E2F target genes, primarily in quiescent cells (Ogawa *et al.*, 2002). The complex contains two MTases together with the transcriptional repressor protein HP1 $\gamma$  (Ogawa *et al.*, 2002). Most interestingly, E2F-6 interacts with members of the Myc family of transcription factors. Both Max and Mga, which form a heterodimer, co-purify with E2F-6 (Ogawa *et al.*, 2002). This result implies that the E2F-6/Max complex could potentially target both E2F and Myc responsive genes (Ogawa *et al.*, 2002). Together with the observation that E2F-6 is replaced by other E2F family members, like E2F-1, as cells move into G1 consolidates the over-riding view that E2F-6 directs transcriptional silencing in quiescent cells by modifying chromatin.

## **1.9 Regulation of E2F activity**

In normal cells E2F activity is tightly regulated during the cell cycle through the temporal association and release of pocket proteins (Dyson, 1998). However, other levels of control exist on E2F activity, such as the association between cyclinA/cdk2 and E2F-1 as cells enter S-phase (Krek *et al.*, 1994; Xu *et al.*, 1994). The function of cyclinA/cdk2 complexes in cell cycle regulated transcription is distinct from that exerted by cyclinE/cdk2 or cyclinD/cdk4.



Binding between E2F-1 and cyclinA/cdk2 results in the phosphorylation of DP-1 and subsequent loss of E2F DNA-binding activity (Krek *et al.*, 1995). This event, in addition to the rapid turnover of cyclinE-mediated by ubiquitin-dependent proteolysis, is required for cell cycle exit from S-phase (Elledge and Harper, 1998). Therefore, whereas cyclins D and E activate E2F-dependent transcription in part by targeting pocket proteins and certain E2F subunits, cyclinA is used to turn off E2F target genes.

Another level of control that impinges on E2F activity includes targeted proteolysis and acetylation. Unphosphorylated pRb can bind to and protect E2F-1 and E2F-4 from ubiquitin-dependent degradation by masking the availability of selected E2F sequences in the C-terminal region (Hateboer *et al.*, 1996; Hofman *et al.*, 1996). The rapid degradation of E2F-1 at the S to G2 transition requires an interaction between E2F-1 and the F-box-containing protein p45<sup>SKP2</sup>, which is the cell cycle-regulated component of the ubiquitin-protein ligase SCF<sup>SKP2</sup> (Marti *et al.*, 1999). Binding of p45<sup>SKP2</sup> to the N-terminal region of E2F-1 in a region distinct from cyclinA targets E2F-1 for degradation (Marti *et al.*, 1999). Therefore, cyclinA regulates the duration of S-phase and the skip/cullin/F-box (SCF) complex targets E2F-1 for destruction once S-phase is complete (Marti *et al.*, 1999). Further, E2F-1 and DP-1 are down-regulated by the murine double minute 2 (MDM2) oncoprotein (Loughran and La Thangue, 2000), which possesses E3 ligase activity (Honda *et al.*, 1997), and p19<sup>ARF</sup> (Martelli *et al.*, 2001; Mason *et al.*, 2002), the gene for which is a transcriptional target of E2F-1 (Bates *et al.*, 1998). The direct regulation of E2F activity is likely to involve multiple processes that ensure E2F activity is tightly regulated throughout the cell cycle.

The transcriptional activity of E2F requires the interaction with the p300/CBP family of co-activator proteins (Trouche *et al.*, 1996; Morris *et al.*, 2001). p300/CBP proteins bind to a diverse array of sequence-specific transcription factors and can simultaneously interact directly with components of the basal transcriptional machinery, such as TFIIB, TBP and RNA polymerase II, and facilitate the assembly of multi-protein cofactor complexes (Chan and La Thangue, 2001). By providing a bridge between transcription factors and the basal transcription machinery, p300/CBP is able to stimulate transcriptional activation (Chan and La Thangue, 2001). p300/CBP proteins possess an intrinsic histone acetyltransferase (HAT) activity and can acetylate histones, a process likely to loosen chromatin structure and cause increased gene expression (Chan and La Thangue, 2001). Additionally, direct acetylation of E2F-1 by the p300/CBP-associated factor (PCAF) occurs at lysine residues within the DNA binding domain, which increases its DNA binding activity, transcriptional activity and protein stability (Martinez-Balbas *et al.*, 2000). Significantly, the acetylated residues are conserved only in the E2F-1 sub-family (Martinez-Balbas *et al.*, 2000). Moreover, pRb is regulated by acetylation (Chan *et al.*, 2001) where adenovirus E1A, which binds p300/CBP through an N-terminal transformation-sensitive domain (Eckner *et al.*, 1994), stimulates the acetylation of pRb by recruiting p300 and pRb into a multi-protein complex (Chan *et al.*, 2001). The acetylation of pRb is cell cycle-regulated and the acetylation of particular lysine residues in the C-terminal region of pRb hinders the subsequent phosphorylation of pRb by cdk complexes (Chan *et al.*, 2001).

## 1.10 Role of E2F in proliferation

One approach to investigate E2F has been to inactivate individual E2F genes (Table 1.2). *E2F-1*<sup>-/-</sup> mice develop normally but exhibit defects in T-cell apoptosis and a broad spectrum of tumours in older mice, suggesting that E2F-1 can under some conditions function as a tumour suppressor (Field *et al.*, 1996; Yamasaki *et al.*, 1996). Mouse embryonic fibroblasts (MEFs) derived from *E2F-1*<sup>-/-</sup> mice show a delayed exit from G0 phase, indicating that E2F-1 has an important role in timing S-phase entry from G0 (Wang *et al.*, 1998). *E2F-3*<sup>-/-</sup> mice arise at one-quarter of the expected frequency, demonstrating that E2F-3 is important for development (Humbert *et al.*, 2000a). Furthermore, *E2F-3*<sup>-/-</sup> MEFs have reduced expression of numerous E2F-responsive genes, including *B-myb*, *cyclinA* and *DHFR* (Humbert *et al.*, 2000a), and micro-injection of anti-E2F-3 antibodies causes cell cycle arrest in primary cells, whilst an E2F-1 antibody has little effect (Leone *et al.*, 1998). In synchronised cells E2F-3 DNA-binding activity accumulates at the G1/S-phase transition of each consecutive cell cycle, whereas E2F-1 only accumulates during the initial G1 following mitogenic stimulus (Leone *et al.*, 1998). Significantly, the combined loss of E2F-1, E2F-2 and E2F-3 is sufficient to completely block cellular proliferation (Wu *et al.*, 2001a). Taken together, these results suggest that E2F-1 and E2F-3 have distinct roles in regulating cell cycle progression: E2F-3 regulates expression of S-phase genes in proliferating cells, and E2F-1 regulates exit from G0.

Interestingly, the *DP-1*<sup>-/-</sup> mouse is embryonic lethal, probably due to defects in proliferation (Kohn *et al.*, 2003).

Whilst *E2F-4*<sup>-/-</sup> mice die *in vivo* and have numerous developmental defects (Rempel *et al.*, 2000; Humbert *et al.*, 2000b), *E2F-5*<sup>-/-</sup> mice are viable but fail to survive to term because of hydrocephaly caused by excessive secretion of cerebral spinal fluid in the choroid plexus (Lindeman *et al.*, 1998). Analysis of MEFs that are derived from E2F and pocket protein knock-out mice, namely *E2F-4*<sup>-/-</sup>, *E2F-4*<sup>-/-</sup>/*E2F-5*<sup>-/-</sup>, or *p107*<sup>-/-</sup>/*p130*<sup>-/-</sup>, has suggested defects in the ability to exit the cell cycle in response to various growth arrest signals, including p16 over-expression (Bruce *et al.*, 2000; Gubatz *et al.*, 2000). Introduction of E2F-4 or E2F-5 into *E2F-4*<sup>-/-</sup>/*E2F-5*<sup>-/-</sup> cells is able to reinstate the p16 response whereas introduction of E2F-1 failed to do so, highlighting the functional differences in these two E2F sub-families (Gubatz *et al.*, 2000). In addition, the combined loss of *p107* and *p130* causes the upregulation of E2F-responsive genes, including *B-myb*, *cdc2* and *E2F-1*, whereas there is little difference in MEFs deficient in either *p107* or *p130* (Hurford *et al.*, 1997). The mutant cells respond appropriately to growth stimulatory signals and show minimal change in their proliferative capacity, suggesting that loss of these E2F/DP-pocket protein complexes impairs mostly the repression of E2F-responsive genes, and therefore the ability to exit the cell cycle (Bruce *et al.*, 2000; Gaubatz *et al.*, 2000). Taken together, these studies establish a link between E2F-4/E2F-5 and the regulation of cell cycle exit and terminal differentiation.

## 1.11 E2F in differentiation

E2F family members have been implicated in the regulation of differentiation. A recent study demonstrated that repression of E2F-1/DP-1 dependent transcription by the C/EBP $\alpha$  transcription factor is essential for cell cycle exit and the differentiation of adipocytes and neutrophil granulocytes *in vivo* (Porse *et al.*, 2001). The importance of repressing E2F activity for the initiation of differentiation has also been observed in myogenic cells and in keratinocytes (Dicker *et al.*, 2000; Wang *et al.*, 1995), and loss of E2F repression through pRb mutation leads to uncontrolled proliferation and apoptosis *in vivo* (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Whilst this highlights the role of both pRb and C/EBP $\alpha$  in repression of E2F activity during development, C/EBP $\alpha$  can repress E2F in the absence of functional pRb protein (Johanson *et al.*, 2001; Slomiany *et al.*, 2000). This suggests that the C/EBP $\alpha$  and pRb pathways of E2F repression act independently. Moreover, in differentiating granulocytic cells p130, rather than pRb, has been found to be the critical pocket protein (Bergh *et al.*, 1999; Mori *et al.*, 1999), indicating that, in addition to E2F-1, repression of E2F-4/-5 is critical for proper differentiation of granulocytes (Porse *et al.*, 2001). The differentiation of pre-adipocytes into adipocytes requires that growth-arrested pre-adipocytes re-enter the cell cycle before undergoing terminal differentiation. Fajas *et al.*, (2002) demonstrated that in confluent pre-adipocytes the E2F-4/p130 complex acts to repress the transcription of E2F target genes, including PPAR $\gamma$ , the master regulator of adipogenesis. Hormonal stimulation results in the loss of this repressive complex and the induction of activating E2Fs,

particularly E2F-1. E2F-1 can directly activate PPAR $\gamma$  transcription, and thereby activate differentiation. Interestingly, during the late stages of differentiation the E2F-4/p130 complex reforms, possibly to switch off PPAR $\gamma$  transcription in terminally differentiated adipocytes.

## 1.12 Role of E2F in cancer

The de-regulation of E2F activity provides tumour cells with a proliferative advantage. E2F activity is disrupted in most if not all tumour cells via activating mutations in the cyclinD1 gene, alteration of the Ink4a gene, or mutation of the pRb gene itself (Sherr, 1996). Elevated levels of cyclinD1 are observed in certain tumour cells due to gene amplification, chromosomal rearrangement (Hall and Peters, 1996) or mutations that prevent normal degradation (Welcker *et al.*, 1996). The p16<sup>Ink4a</sup> gene is frequently found to be mutated in human tumours (Koh *et al.*, 1995) and a missense mutant cdk4, which is unable to associate with p16, renders cdk4 constitutively active (Wolfel *et al.*, 1995). The fact that multiple mutations in these genes do not occur in a single tumour suggests that inactivation of any one of these components in the pRb pathway is sufficient to favour tumourigenesis (Yamasaki, 1999). Interestingly, E2F is not a frequent target of mutations in cancer (Dyson, 1998), indicating that it is the inactivation of the pathway with subsequent de-regulation of E2F, rather than the direct mutagenic activation of E2F, that is a hallmark of tumourigenesis.

### 1.13 E2F-1: Oncogene or tumour suppressor

It appears that the level of expression and the cellular environment influences whether E2F-1 can participate in the process of oncogenesis or tumour suppression (Pierce *et al.*, 1999). E2F-1 possesses oncogenic properties both *in vitro* and *in vivo*, and E2F-1 over-expression is sufficient to induce the transformation of primary cells (Johnson *et al.*, 1994), whilst the over-expression of E2F-1 in transgenic mice promotes tumourigenesis (Pierce *et al.*, 1999). Tumours in mice where E2F-1 activity is compromised grow more slowly than their wild-type counterparts (Yamasaki *et al.*, 1998).

Nevertheless, in addition to its role in proliferation, E2F-1 can trigger apoptosis, and it is through this ability that E2F-1 may act as a tumour suppressor. *E2F-1*<sup>-/-</sup> mice exhibit defects in apoptosis, together with an increased incidence of tumours (Field *et al.*, 1996; Yamasaki *et al.*, 1996), providing evidence that E2F-1 possesses tumour suppressor activity *in vivo*. In addition, the aberrant apoptosis and S-phase entry observed in mice carrying homozygous mutation of Rb is suppressed by E2F-1 deficiency (Tsai *et al.*, 1998). E2F-3 deficiency also suppresses inappropriate apoptosis in Rb mutant mice embryos, providing evidence that E2F-3, in addition to E2F-1, plays a role in the induction of apoptosis that arises from Rb deficiency (Saavedra *et al.*, 2002; Ziebold *et al.*, 2001). Interestingly, the induction of apoptosis by c-Myc is E2F-1 dependent and is inhibited in cells lacking E2F-1, but not E2F-2 or E2F-3 (Leone *et al.*, 2001). However, the ability of Myc to induce S-phase is impaired in the absence of E2F-2 or E2F-3, but not E2F-1. Therefore it is likely that

different E2F activities control cell proliferation and cell death decisions (Leone *et al.*, 2001).

E2F-1 can trigger apoptosis through both p53-dependent and independent pathways, and although transcription-independent functions of E2F-1 have been ascribed to apoptosis (Hsieh *et al.*, 1997; Phillips *et al.*, 1997; Phillips *et al.*, 1999), a number of genes involved in apoptosis are regulated at the transcriptional level by E2F-1. Thus, E2F-1 augments p53-dependent apoptosis through the transcriptional activation of the p19<sup>ARF</sup> tumour suppressor gene (Bates *et al.*, 1998). ARF sequesters the E3 ubiquitin ligase MDM2 into the nucleolus, thereby preventing MDM2 from targetting p53 for ubiquitination and subsequent degradation (Weber *et al.*, 1999). Therefore, the induction of ARF by E2F-1 leads to p53 stabilisation and activation. However, E2F-1 can induce p53-dependent apoptosis in the absence of ARF (Russel *et al.*, 2002; Tolbert *et al.*, 2002; Tsai *et al.*, 2002), and loss of ARF can enhance E2F-1 induced apoptosis (Russel *et al.*, 2002; Tsai *et al.*, 2002). In support of these observations, over-expression of ARF can inhibit E2F-1-dependent apoptosis (Mason *et al.*, 2002), and targets E2F-1 for proteasomal degradation (Martelli *et al.*, 2001). In addition, E2F-1 can interact with p53 directly via the cyclinA binding domain of E2F-1, and the interaction enhances p53 apoptotic activity (Hsieh *et al.*, 2002).

E2F-1 can signal apoptosis independently of p53 via direct transcriptional activation of the p53 family member p73 (Irwin *et al.*, 2000; Stiewe *et al.*, 2000), and the physiological apoptotic process called TCR-activation-induced cell death (TCR-AICD), requires both E2F-1 and p73 (Lissey *et al.*, 2000).



The Apaf-1 gene, which mechanistically regulates cytochrome c release from mitochondria leading to caspase activation, provides another direct target for E2F-1 (Moroni *et al.*, 2001; Furukawa *et al.*, 2002). Consistent with this, E2F-1 induced apoptosis is significantly compromised by inhibition of caspase activity or by disruption of the Apaf-1 gene (Moroni *et al.*, 2001; Furukawa *et al.*, 2002). In addition, analysis of *Apaf-1<sup>-/-</sup>/Rb<sup>-/-</sup>* mouse embryos demonstrates that Apaf-1 is required for apoptosis induced by Rb deficiency in some tissues (Guo *et al.*, 2001).

E2F-1 has also been shown to participate directly in apoptotic pathways by stimulating the accumulation of caspases through a direct transcriptional mechanism (Nahle *et al.*, 2002).

E2F-1 can also promote apoptosis by inhibiting NF- $\kappa$ B activity. The NF- $\kappa$ B transcription factor regulates cell survival by activating various anti-apoptotic genes (Karin *et al.*, 2002). By down regulating TRAF-2 protein levels, E2F-1 impairs NF- $\kappa$ B activation in response to tumour necrosis factor  $\alpha$  (TNF $\alpha$ ; Philips *et al.*, 1999). In addition, E2F-1 can inhibit NF- $\kappa$ B DNA-binding activity providing another mechanism of inhibition of NF- $\kappa$ B activity by E2F-1 (Tanaka *et al.*, 2002).

Consistent with E2F-1's role in inhibition of anti-apoptotic signals, exploring E2F target gene specificity using DNA microarrays has revealed that E2Fs up-regulate the expression of pro-apoptotic members of the Bcl-2 family, including Bok, Bad, Bak and Bid-1 (Ma *et al.*, 2002; Polager *et al.*, 2002; Stanelle *et al.*, 2002).

## 1.14 E2F-1 is induced in response to DNA damage

Overexpression of E2F-1 sensitises cells to apoptosis in response to DNA damage caused by ionising radiation (IR), or chemotherapeutic drugs such as the topoisomerase II inhibitors etoposide and adriamycin (Nip *et al.*, 1997; Pruschy *et al.*, 1999). Recent studies have found a direct role for E2F-1 in the DNA damage response pathway, as treating cells with DNA damaging agents increases E2F-1 protein levels and DNA-binding activity (Blattner *et al.*, 1999; Hofferer *et al.*, 1999; O'Connor *et al.*, 2000; Lin *et al.*, 2001). It has been suggested that ataxia telangiectasia mutated (ATM) phosphorylates and stabilises E2F-1 in response to DNA damage (Lin *et al.*, 2001), through a mechanism that perhaps involves the ubiquitin ligase p45<sup>SKP2</sup> (Marti *et al.*, 1999), or the BRCT-domain containing protein DNA topoisomerase II $\beta$  binding protein 1 (TopBP1; Liu *et al.*, 2003).

## 1.15 Cell cycle checkpoints

With every cell-division cycle, the cell must duplicate the genome and segregate the identical sets of chromosomes into two daughter cells. It is imperative that these events are carried out accurately and efficiently, to ensure this, cells have developed mechanisms that are known as the cell cycle checkpoints. Checkpoints detect incompletely replicated or damaged DNA (DNA replication and DNA damage checkpoints respectively), and transmit a signal to effector molecules that stall the cell cycle to allow time for DNA to be repaired, or to activate programmed cell death. Checkpoint pathways also co-

ordinate the resumption of cell cycle progression, provided replication is complete and DNA lesions have been repaired.

The cell cycle can potentially arrest at several stages, but it usually arrests prior to S-phase (G1 checkpoint), during S-phase (intra-S-phase checkpoint) or before mitosis (the G2 checkpoint; Figure 1.3).

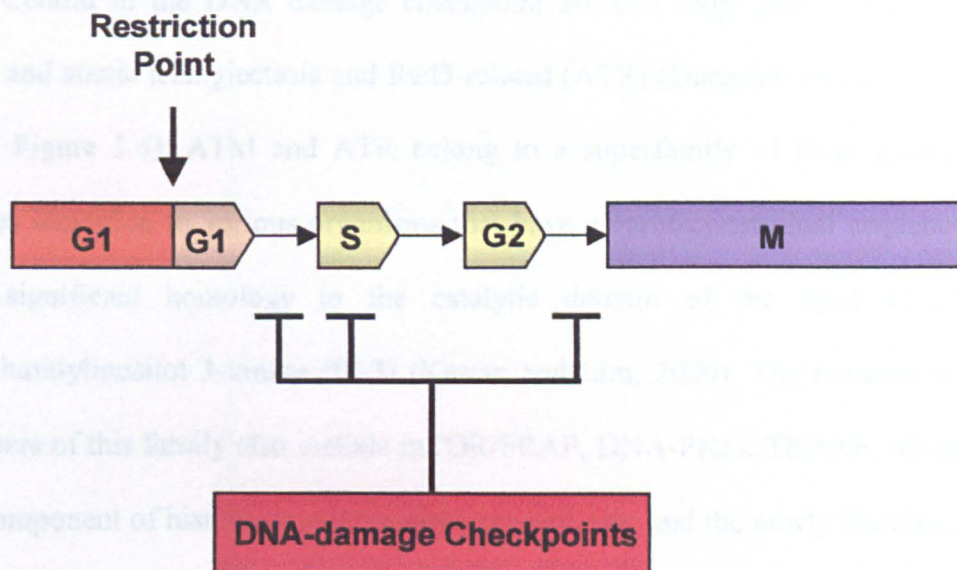
Arrest in G1 delays the start of S-phase and thus prevents the replication of a damaged DNA template. However, G1 cells only contain one copy of the genome, therefore repair of the damaged DNA cannot be achieved via homologous recombination. Rather cells must use the non-homologous end-joining (NHEJ) pathway, in which two DNA ends that share little or no sequence homology are joined. This might explain why the G1 checkpoint is closely linked to apoptosis.

The intra-S-phase checkpoint reduces the rate of DNA replication without completely blocking progression through S-phase, presumably to afford time for DNA to be repaired.

The G2/M checkpoint provides time for the repair of damaged chromatids prior to their segregation in mitosis. G2 cells contain two full complements of genetic material, therefore DNA damage can be repaired by homologous recombination, without loss or change of genetic information.

## **1.16 DNA damage signalling pathway**

DNA damage poses a considerable threat to genomic integrity and cell survival. DNA damage arises spontaneously, for example during DNA replication, and in response to exogenous agents, such as IR.



**Figure 1.3 Cell cycle checkpoints**

The DNA damage checkpoints can arrest the cell prior to S-phase (G1 checkpoint), during S-phase (intra S-phase checkpoint) or before mitosis (the G2 checkpoint). Checkpoints allow cell cycle progression to be modulated even after the restriction point has been traversed, after which the cell progresses through the cycle in an autonomous manner. Arrest in G1 prevents the replication of a damaged DNA template, and is closely linked to apoptosis. The intra S-phase checkpoint reduces the rate of DNA replication, but does not completely stop the cell cycle. The G2 checkpoint prevents the segregation of damaged chromatids.

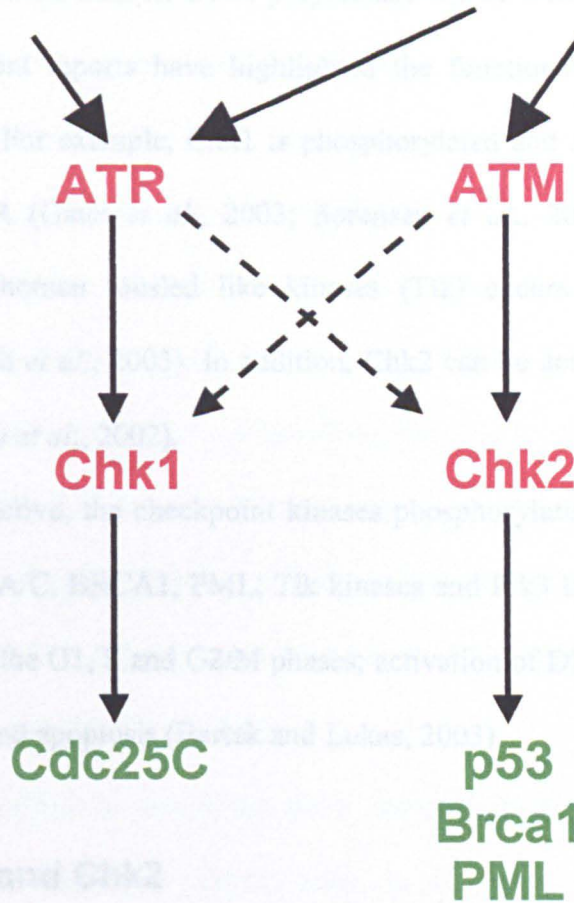
Although significant progress is being made, it is not fully understood how DNA damage is detected, but DNA lesions, such as double-stranded (d/s) breaks, leads to the rapid activation of a multi-protein cascade collectively known as the DNA damage signalling pathway.

Central to the DNA damage checkpoint are two large protein kinases, ATM and ataxia telangiectasia and Rad3-related (ATR) (Durocher and Jackson, 2001; Figure 1.4). ATM and ATR belong to a superfamily of large protein kinases identified in various organisms that have a carboxy-terminal sequence with significant homology to the catalytic domain of the lipid kinase phosphatidylinositol 3-kinase (PI-3) (Kastan and Lim, 2000). The mammalian members of this family also include mTOR/FRAP, DNA-PKcs, TRRAP, which is a component of histone acetyltransferase complexes, and the newly identified ATX which likely contributes to checkpoint activation by diverse types of genotoxic stress (Shiloh, 2003).

ATM and ATR are at the top of the signalling pathways induced by DNA damage. Evidence for this was derived from studies using cells isolated from patients with the disorder AT (ataxia telangiectasia). AT cells lack the ATM protein, and are defective in all three DNA damage checkpoint responses (McGowan, 2002). ATM and ATR respond to DNA damage by phosphorylating and activating two downstream effector kinases, known as checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2; Figure 1.4). The original model suggested that the ATM-Chk2 pathway is activated by the generation of DNA d/s breaks that are induced by ionising radiation or radiomimetic drugs. The ATR-Chk1 pathway, by contrast, is activated in response to stalled replication forks that are induced by either UV-irradiation

**Replication inhibition**

**Ionising Radiation**



**Figure 1.4 The DNA damage signalling pathway**

Schematic representation of the mammalian DNA damage signalling pathway. Central to the DNA damage checkpoint are two large protein kinases, ATM and ATR. ATM and ATR respond to DNA damage by phosphorylating and activating two downstream effector kinases, Chk1 and Chk2. It is not yet fully understood how DNA damage is detected, but the original model suggested that the ATM-Chk2 pathway is activated by the generation of DNA double stranded breaks that are induced by ionising radiation or radiomimetic drugs. The ATR-Chk1 pathway, by contrast, is activated in response to stalled replication forks (caused by UV, hydroxyurea and aphidicolin), as well as DNA double stranded breaks. However, recent reports have highlighted the functional redundancy between these kinases. The checkpoint kinases then phosphorylate target proteins such as p53, Cdc25C, BRCA1 and PML to arrest cell cycle progression, activate DNA repair pathways, or induce apoptosis.

(which stalls replication by generating nucleotide dimers) or drugs such as hydroxyurea (which depletes the cellular deoxyribonucleotide pool) and aphidocolin (which inhibits DNA polymerase  $\alpha$ ), as well as DNA d/s breaks. However, recent reports have highlighted the functional redundancy among these kinases. For example, Chk1 is phosphorylated and activated by ATM in response to IR (Gatei *et al.*, 2003; Sorensen *et al.*, 2003), and checkpoint signalling to human touselled like kinases (Tlk) occurs via an ATM-Chk1 pathway (Groth *et al.*, 2003). In addition, Chk2 can be activated independently of ATM (Hirao *et al.*, 2002).

Once active, the checkpoint kinases phosphorylate target proteins such as p53, Cdc25A/C, BRCA1, PML, Tlk kinases and Plk3 kinases, to cause cell-cycle arrest in the G1, S and G2/M phases; activation of DNA repair; chromatin restructuring and apoptosis (Bartek and Lukas, 2003).

## 1.17 Chk1 and Chk2

Chk1 and Chk2 are structurally distinct Ser/Thr kinases that share overlapping substrate specificity *in vitro* (Bartek *et al.*, 2001) and may share some functions *in vivo*.

There are, however, some important differences between Chk1 and Chk2. For example, Chk2 is a relatively stable protein (half-life greater than six hours), which is expressed and can be activated in all phases of the cell cycle, including G0 (Lukas *et al.*, 2001). Moreover, Chk2 is present and can be activated in some differentiated cells and tissues (Lukas *et al.*, 2001). By contrast, Chk1 is an unstable protein (half-life less than two hours), and its



expression is restricted to S and G2 phases of the cell cycle (Lukas *et al.*, 2001; Kaneko *et al.*, 1999). Chk1 is also absent in differentiated cells (Lukas *et al.*, 2001). The observation that Chk1 expression is restricted to S and G2 phases of proliferating cells suggests that DNA damage signalling in G1, and G0, may depend exclusively on Chk2.

The tumour suppressor protein p53 can down-regulate the expression of Chk1, but not Chk2, dependent on the transcriptional activity of p53, and the presence of functional p21 (Gottifredi *et al.*, 2001). The tumour suppressor protein pRb is also required for Chk1 down-regulation, suggesting the possible involvement of E2F-dependent transcription in the regulation of Chk1 (Gottifredi *et al.*, 2001). Consistent with this idea, chromatin immunoprecipitation (Chip) assays have shown E2F family members bound to the Chk1 promoter (Ren *et al.*, 2002).

The analysis of knock-out mice carrying inactivated Chk1 or Chk2 genes indicates they play distinct roles in development. Mice harbouring homozygous disruption of Chk2 are viable (Hirao *et al.*, 2000; Takai *et al.*, 2002; Jack *et al.*, 2002), however homozygous disruption of Chk1 causes embryonic lethality (Liu *et al.*, 2000; Takai *et al.*, 2000). In contrast, Chk1 deficiency in avian somatic DT-40 lymphoma cells can be tolerated, but interferes with normal DNA damage responses, and cell survival after IR (Zachos *et al.*, 2003).



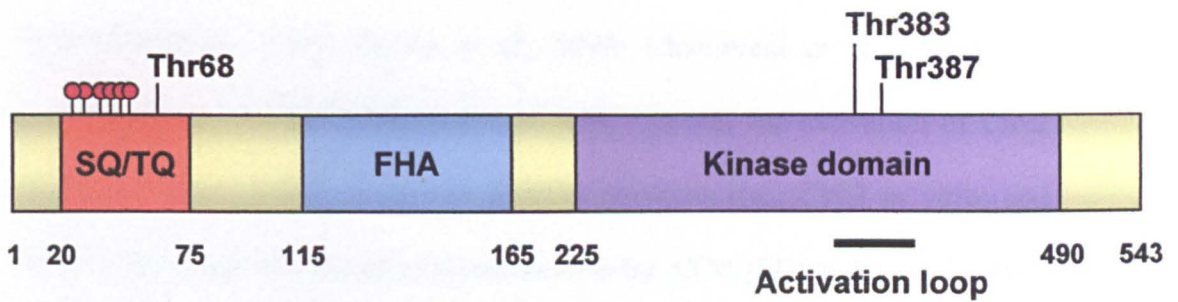
## 1.18 Chk2 structure and activation

The human Chk2 gene encodes a 543 amino acid homologue of the *Saccharomyces cerevisiae* Rad53 and *Schizosaccharomyces pombe* Cds1 checkpoint kinases (Matsuoka *et al.*, 1998). The overall structure of the Chk2 proteins is similar in all eukaryotes, and the degree of overall homology of Chk2 protein sequences across species roughly reflects the evolutionary distance among the distinct organisms. For example, human Chk2 shows 83% and 82% amino-acid identity with the rat and mouse kinases; 61% identity with the *Xenopus laevis* protein; 51% with the zebrafish; 34% with *Drosophila melanogaster*; 32% with both *S. pombe* and the nematode *Caenorhabditis elegans*; and 28% with *S. cerevisiae* Rad53 (Bartek *et al.*, 2001).

At the protein level the structure of human Chk2 is dominated by three evolutionarily conserved domains (Figure 1.5). An SQ/TQ cluster domain (SCD) is located at the N-terminus (residues 20-75), a forkhead-associated domain (FHA) within the central portion (residues 115-165), and a kinase domain within the C-terminal region (residues 225-490).

The kinase domain is very large, occupying almost the entire carboxy-terminal half of Chk2. Its key functional elements - including the activation loop - have been identified based on their homology with other Ser/Thr kinases. A kinase-defective, dominant-negative mutant, has been generated by mutating one of the key residues of this domain, Asp347Ala (Matsuoka *et al.*, 1998).

The SCD located in the amino-terminal domain of Chk2 contains a series of seven serine or threonine residues followed by glutamine (SQ or TQ motifs). These motifs are the preferred sites for phosphorylation by ATM/ATR



**Figure 1.5 Structure of the Chk2 protein**

Structure of the human Chk2 protein, highlighting the positions of the SQ/TQ-rich (SCD), forkhead-associated (FHA), and kinase domains. Closed lollipops indicate sites phosphorylated by ATM/ATR kinases. Phosphorylation sites at Thr68 (ATM/ATR) and Thr383 and Thr387 (auto-phosphorylation) involved in the activation of Chk2 are indicated. (Figure adapted from Bartek *et al.*, 2001).

kinases (Kastan and Lim, 2000). In normal cells, Chk2 is rapidly phosphorylated and activated in response to replication blocks and DNA damage (Matsuoka *et al.*, 1998; Brown *et al.*, 1999; Chaturvedi *et al.*, 1999).

The response to DNA damage caused by IR occurs in an ATM-dependent manner as Chk2 is not activated in cells derived from AT patients (Matsuoka *et al.*, 1998; Brown *et al.*, 1999; Chaturvedi *et al.*, 1999), and transfection of AT cells with functional ATM restores the activation of Chk2 (Matsuoka *et al.*, 1998). ATM can directly phosphorylate Chk2 *in vitro*, and Thr68 is the major site phosphorylated *in vitro* by ATM (Matsuoka *et al.*, 2000; Melchionna *et al.*, 2000; Ahn *et al.*, 2000). Moreover, *in vivo* Thr68 is phosphorylated in an ATM-dependent manner in response to IR (Matsuoka *et al.*, 2000; Melchionna *et al.*, 2000; Ahn *et al.*, 2000). The importance of this phosphorylation site has been demonstrated by the failure of mutant, non-phosphorylatable forms of Chk2 to be activated *in vivo* (Matsuoka *et al.*, 2000; Melchionna *et al.*, 2000; Ahn *et al.*, 2000).

In response to UV-irradiation or HU treatment, Chk2 is phosphorylated and activated in an ATM-independent manner (Matsuoka *et al.*, 1998; Brown *et al.*, 1999; Chaturvedi *et al.*, 1999). ATR can also phosphorylate Chk2 on Thr68 *in vitro*, and phospho-specific antibodies confirm that Thr68 is phosphorylated in response to UV-irradiation and HU in cells lacking ATM (Matsuoka *et al.*, 2000). Phosphorylation of Thr68 in response to IR does occur, but with delayed kinetics in cells lacking ATM (Matsuoka *et al.*, 2000). Interestingly, phosphorylation of Ser15 on p53, the site phosphorylated by ATM and ATR *in vitro* (Canman *et al.*, 1998; Banin *et al.*, 1998; Tibbetts *et al.*, 1999), is also delayed in response to IR in cells lacking ATM (Siliciano *et al.*, 1997). It has

been suggested that ATR phosphorylates Ser15 in late phase in response to IR (Tibbetts *et al.*, 1999). Therefore, ATR is likely to phosphorylate Thr68 on Chk2 *in vivo* in response to UV-irradiation and HU, and in late phase in response to IR.

Phosphorylation at Thr68 is a prerequisite for the subsequent activation step, which is attributable to auto-phosphorylation of Chk2 on residues Thr383 and Thr387 in the activation loop of the kinase domain (Lee *et al.*, 2001; Ahn *et al.*, 2002). Both Thr383 and Thr387 are phosphorylated *in vivo* following IR, and mutation of these sites results in a catalytically inactive Chk2 (Lee *et al.*, 2001). It has been proposed that the activating auto-phosphorylation of Chk2 on Thr383 and Thr387 depends on the integrity of another key domain, the FHA domain (Lee *et al.*, 2001; Ahn *et al.*, 2002). Consistent with this, a tumour-derived Chk2 mutation within the FHA domain, Phe145Trp, destabilises the structure of the FHA domain and renders the protein incapable of being Thr68 phosphorylated and activated in an ATM-dependent manner (Lee *et al.*, 2001; Li *et al.*, 2001; Wu *et al.*, 2001; Falck *et al.*, 2001a). Furthermore, the identification of mutations within the FHA domain of Chk2 in several variant Li-Fraumeni syndrome (LFS) families and sporadic cancers strengthens the concept that this domain is important for Chk2 function (Bell *et al.*, 2000; Miller *et al.*, 2002; Lee *et al.*, 2001).

FHA domains recognise phosphorylated Ser/Thr motifs and function to mediate protein–protein interactions (Durocher and Jackson, 2002). Therefore the FHA domain may mediate the docking of Chk2 onto ATM or other proteins involved in cell cycle checkpoint signalling. In support of this idea, phosphorylation of Thr68 promotes oligomerisation of Chk2 via binding of the

Thr68 phosphorylated region of one Chk2, to the FHA domain of another (Ahn *et al.*, 2002). The dimers formed following DNA damage are transient in nature during activation, and trans-auto-phosphorylation at multiple sites including Thr383 and Thr387 is proposed to release active Chk2 monomers that proceed to enforce checkpoint control (Ahn *et al.*, 2002). Consistent with these findings, Xu *et al.*, (2002) show that both the SCD and FHA domains are required for formation of active Chk2 oligomers, and that Thr68 Chk2 phosphorylation is required for stable dimerization. Finally, a recent study has suggested that while Thr68 phosphorylation is required for oligomerization and activation of Chk2, it is not required for the maintenance of dimerization or kinase activity (Ahn and Prives, 2002).

Taken together, these studies point towards a model in which inter-molecular auto-phosphorylation of Thr383 and Thr387 and subsequent activation of Chk2 occurs, providing a very quick, powerful mechanism of signal transduction.

## **1.19 Mediators of Chk2 activation**

In budding yeast, phosphorylation and activation of the Chk2 homologue Rad53 by Mec1 (the homologue of ATM) requires Rad9, a protein with C-terminal tandem BRCT (BRCA1 C-terminus) motifs. Recently, a group of large BRCT domain containing proteins, including 53BP1, NFB1/MDC1, and BRCA1 have been identified as mediators of checkpoint responses in mammalian cells.

53BP1, a protein identified through its ability to bind to the tumour suppressor protein p53 via 53BP1's C-terminal BRCT repeats (Iwabuchi *et al.*, 1994), resembles the Rad9 BRCT-repeat protein in budding yeast. In response to IR, Chk2 is phosphorylated on Thr68, and co-localises within discrete nuclear foci along with 53BP1 (Zhou and Elledge, 2000; Ward *et al.*, 2001). In cells where 53BP1 is inhibited with siRNA, a reduction, but not abrogation, of Chk2 Thr68 phosphorylation and foci formation is observed in response to IR, suggesting that 53BP1 plays a partially redundant role in the activation of Chk2 (Wang *et al.*, 2002). Antibodies to Chk2 efficiently immunoprecipitate 53BP1, however Chk2 dissociates from 53BP1 in response to IR (Wang *et al.*, 2002). This suggests that 53BP1 may act as an adapter that facilitates Chk2 phosphorylation and activation in a transient complex and, upon activation, Chk2 dissociates from the 53BP1 complex. Further, mice in which the 53BP1 gene is disrupted feature a defective DNA damage response with impaired Chk2 activation after low doses of IR (Ward *et al.*, 2003), providing physiological evidence that 53BP1 is required for proper activation of Chk2.

BRCA1, another recently identified substrate of Chk2 (Lee *et al.*, 2000), is one of the key checkpoint controlling proteins (Scully and Livingston, 2000). Human Chk2 and BRCA1 exist within overlapping nuclear foci (Lee *et al.*, 2000). In response to IR Chk2 phosphorylates BRCA1 on Ser988, leading to the release of BRCA1 from its complex with Chk2, and this event is important for BRCA1 to restore cell survival after DNA damage (Lee *et al.*, 2000). BRCA1 has been proposed as a master molecule that assembles and integrates the many components of the early checkpoint response into a dynamic multi-component complex called the BASC (BRCA1-associated genome surveillance complex)

(Wang *et al.*, 2000). It remains to be seen whether Chk2 is a genuine component of the BASC, however BRCA1 and Chk2 do physically interact (Lee *et al.*, 2000), and this might bring Chk2 in close proximity to the upstream ATM/ATR kinases that are required to initiate Chk2 activation (Bartek *et al.*, 2001).

Alternatively, BRCA1 might operate in a similar manner to yeast Rad9 and assemble Chk2 molecules into oligomers to promote inter-molecular auto-phosphorylation, which is required for its full activation and efficient amplification of the checkpoint signalling cascade (Bartek *et al.*, 2001). This scenario is complicated further by the observation that BRCA1 and its regulatory partner CtIP are phosphorylated in response to DNA damage by ATM/ATR and ATM kinases respectively (Li *et al.*, 2000; Cortez *et al.*, 1999; Tibbets *et al.*, 2000).

Interestingly, ATM-dependent activation of Chk2 by IR requires Nbs1 (Buscemi *et al.*, 2001), a component of the Mre11/Rad50/Nbs1 complex. In Nijmegen breakage syndrome (NBS) cells, null for Nbs1 protein, Chk2 phosphorylation and activation are both defective. Importantly, these defects in NBS cells can be complemented by re-introduction of wild-type Nbs1, but not by a C-terminal deleted mutant of Nbs1 unable to form a complex with Mre11 and Rad50, or by an Nbs1 mutated at the ATM phosphorylation site. These findings suggest a role for functional Nbs1 and Mre11 complex in Chk2 activation.

NFBD1 (nuclear factor with BRCT domains protein 1), also called MDC1, binds to the Mre11/Rad50/Nbs1 complex (Goldberg *et al.*, 2003), and has been identified as an early participant in damage induced nuclear foci containing the histone H2A variant H2AX, 53BP1, BRCA1 the

Mre11/Rad50/Nbs1 complex and Chk2 (Peng *et al.*, 2003; Lou *et al.*, 2003; Goldberg *et al.*, 2003; Stewart *et al.*, 2003). MDC1 is phosphorylated in an ATM and Chk2 dependent manner after DNA damage, and suppression of MDC1 expression results in a defective S-phase checkpoint, reduced apoptosis, and decreased p53 stabilisation in response to DNA damage (Lou *et al.*, 2003; Goldberg *et al.*, 2003; Stewart *et al.*, 2003). These defects can be restored by the expression of wild-type MDC1, but not MDC1 with a deleted FHA domain (Lou *et al.*, 2003; Goldberg *et al.*, 2003; Stewart *et al.*, 2003). The FHA domain of MDC1 mediates its association with the phosphorylated Thr68 residue of Chk2 (Luo *et al.*, 2003), therefore these results suggest that MDC1 is recruited via its FHA domain to activated Chk2, and plays a critical role in Chk2 mediated DNA damage responses. A partially redundant role for MDC1 in regulating the phosphorylation of Thr68 and activation of Chk2 is suggested by the fact that siRNA directed against MDC1 in human cells diminishes, but does not abrogate, Thr68 Chk2 in IR-induced foci (Peng *et al.*, 2003).

Taken together, these studies suggest roles for 53BP1, BRCA1, NFB1/MDC1 and the Mre11/Rad50/Nbs1 in the regulation of Chk2 activation, possibly to modulate diverse checkpoint events.

Consistent with the fact that some substrates of Chk2 are soluble, mobile proteins, live cell imaging of Chk2 in human cells demonstrated that Chk2 is also a mobile protein that is redistributed throughout the nucleus in response to DNA damage (Lukas *et al.*, 2003). These results support a role for Chk2 in spreading the checkpoint signal from sites of DNA damage to effector proteins throughout the cell. Although these results are inconsistent with other reports that activated Chk2 forms nuclear foci in response to DNA damage, it remains



possible that Chk2 first needs to localise with other checkpoint proteins in order to become activated.

Finally, an apparently unique feature of mammalian Chk2 that is not conserved in lower eukaryotes is a c-Abl SRC homology domain-consensus binding sequence, which is upstream of the FHA domain (Brown *et al.*, 1999). C-Abl is also phosphorylated and activated by ATM in response to DNA damage, therefore it is possible that Chk2 might also interact with c-Abl, either physically or functionally to mediate checkpoint responses (Bartek *et al.*, 2001).

The emerging role of these BRCT domain containing proteins is to modulate diverse checkpoint events, including activation of Chk1 and Chk2. By regulating protein-protein interactions, these mediators may control the timing and extent of Chk2 activation.

## **1.20 The G1 Checkpoint**

Arrest of the cell cycle at the G1 checkpoint is caused by DNA damage, which causes increased cellular levels of the p53 tumour suppressor. p53 is a transcription factor that induces the expression of a number of gene products whose function is either to arrest cell growth or promote apoptosis (Hickman *et al.*, 2002). The downstream mediators of these p53-regulated cellular effects include the p21 inhibitor of cdks, apoptosis promoting factors such as Bax and Fas, and other targets whose roles in response to DNA damage are not yet known (Hickman *et al.*, 2002). Activation of p53 involves phosphorylation of the transactivation domain, and both acetylation and phosphorylation of the basic allosteric control region (Lakin and Jackson, 1999).

IR induces phosphorylation of p53 on Ser15 (Siliciano *et al.*, 1997) and this phosphorylation is delayed in ATM deficient cells (Siliciano *et al.*, 1997). It has been suggested that ATR phosphorylates Ser15 in late phase in response to IR (Tibbets *et al.*, 1999), and *in vitro* kinase assays confirmed that ATM and ATR directly phosphorylate p53 on the same site, Ser15 (Canman *et al.*, 1998; Banin *et al.*, 1998; Tibbets *et al.*, 1999). However, mutation of Ser15 to Ala, preventing the phosphorylation of p53, has little effect on the half-life of p53 or its ability to cause cell cycle arrest or apoptosis (Ashcroft *et al.*, 1999; Chehab *et al.*, 1999). In fact, phosphorylation of Ser15 seems to affect the ability of p53 to bind to the transcriptional co-activator p300, which modulates the transactivation of target genes by p53 (Dumaz and Meek, 1999).

IR induces phosphorylation of another serine residue in p53, Ser20, in an ATM dependent manner (Chehab *et al.*, 1999; Shieh *et al.*, 1999). Interestingly, Ser20 is in the middle of the domain that binds to MDM2, an important regulator of p53 degradation (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997), and mutation of this site abrogates p53 stabilisation in response to DNA damage (Chehab *et al.*, 1999). Neither ATM or ATR can phosphorylate Ser20 *in vitro* (Canman *et al.*, 1998; Banin *et al.*, 1998), so Ser20 is phosphorylated after IR by some other kinase that is dependent on ATM.

Evidence suggests that in human cells ATM functions upstream of Chk2. ATM phosphorylates Chk2 at Thr68 *in vitro*, and IR leads to Chk2 phosphorylation at Thr68 and its subsequent activation in wild-type but not ATM deficient cells (Matsuoka *et al.*, 2000; Melchionna *et al.*, 2000; Ahn *et al.*, 2000). *In vitro*, Chk2 phosphorylates p53 on Ser20 (Chehab *et al.*, 2000; Shieh *et al.*, 2000), and expression of a kinase defective form of Chk2 (Asp347Ala)

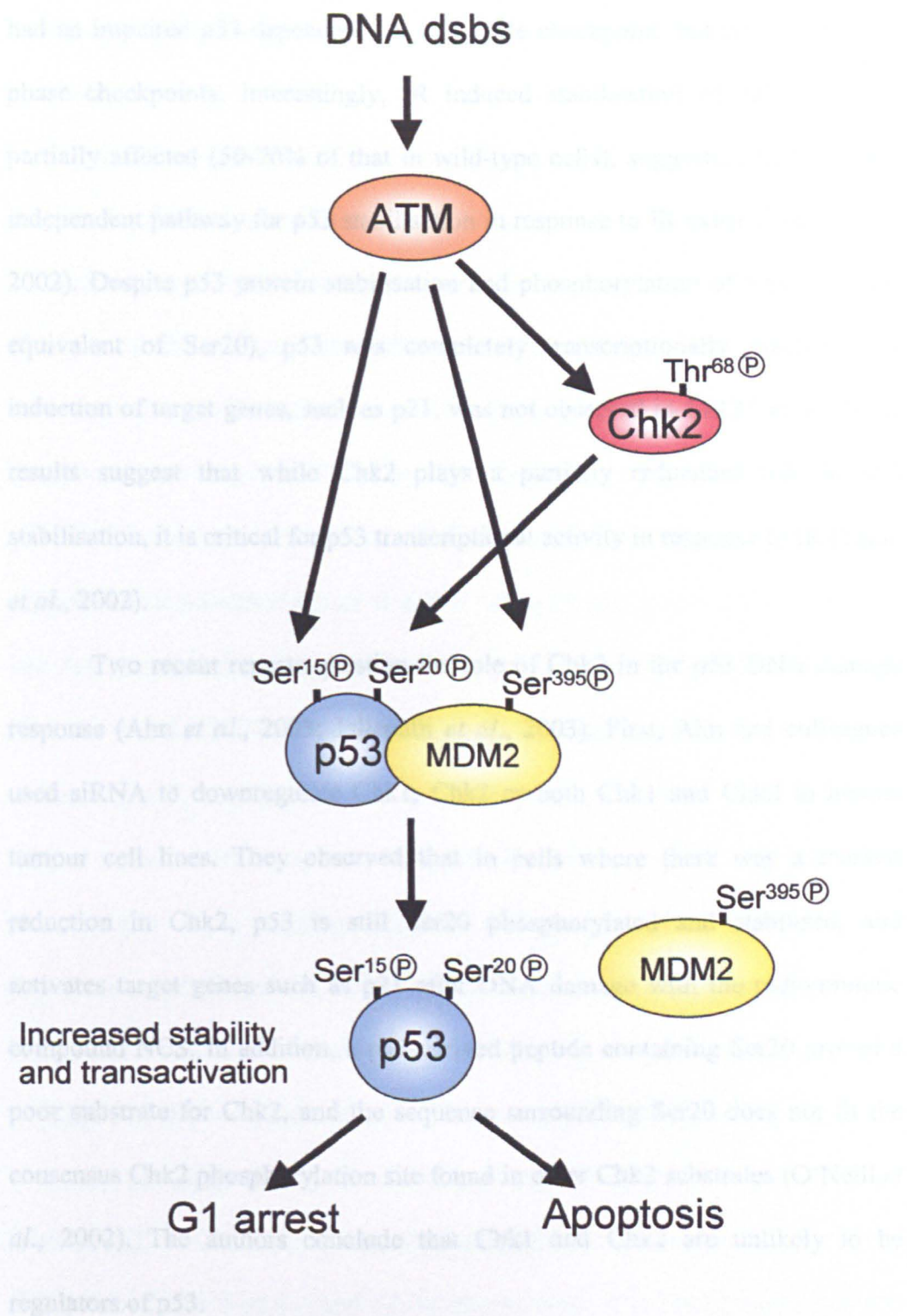
prevents the stabilisation and phosphorylation at Ser20 of p53 in response to IR (Chehab *et al.*, 2000). Moreover, phosphorylation of p53 on Ser20 by Chk2 was shown to dissociate pre-formed p53/MDM2 complexes *in vitro* (Chehab *et al.*, 2000). Taken together, these studies suggest that phosphorylation of p53 on Ser20 by Chk2 leads to p53 protein stabilisation by preventing its interaction with MDM2 (Figure 1.6).

Similarly, Chk1 can phosphorylate p53 on Ser20 *in vitro*, and expression of a kinase defective Chk1 or anti-sense Chk1 leads to a reduction of p53 levels (Shieh *et al.*, 2000).

The analysis of mouse *Chk2*<sup>-/-</sup> deficient cells further supports a role for Chk2 as being upstream of p53. Mouse *Chk2*<sup>-/-</sup> deficient cells that are exposed to IR (but not UV-irradiation) fail to stabilise and activate p53, and are defective in the induction of p53-dependent transcripts such as p21 (Hirao *et al.*, 2000). *Chk2*<sup>-/-</sup> cells exposed to IR also fail to maintain arrest in the G2 phase of the cell cycle, and are resistant to DNA damage induced apoptosis (Hirao *et al.*, 2000).

Another group using MEFs derived from the same mice reported that G1 arrest and p21 induction in response to IR was normal, however the cells failed to undergo DNA damage induced apoptosis (Jack *et al.*, 2002). In the same study, *ATM*<sup>-/-</sup> cells behaved as normal cells in invoking an apoptotic response, therefore the authors conclude that Chk2 is not involved in ATM and p53 dependent G1 arrest, but is involved in the activation of p53, independently of ATM, in triggering DNA damage induced apoptosis.

Results from a second, independently generated *Chk2*<sup>-/-</sup> mouse indicate that Chk2 loss protects mice from IR induced death due to reduced apoptosis in a variety of tissues (Takai *et al.*, 2002). MEFs derived from these *Chk2*<sup>-/-</sup> mice



**Figure 1.6 Role of ATM and Chk2 at the G1 checkpoint**

In response to DNA double stranded breaks, ATM regulates p53 accumulation directly by phosphorylating p53 on Ser15, and MDM2 on Ser395. ATM can regulate p53 accumulation indirectly through the activation of Chk2, which phosphorylates p53 on Ser20. Combined, these multiple phosphorylations lead to the disruption of the p53-MDM2 interaction leading to p53 stabilisation, and increase the transactivating function of p53 resulting in cell cycle arrest or apoptosis.

had an impaired p53-dependent G1 cell cycle checkpoint, but not G2/M or S-phase checkpoints. Interestingly, IR induced stabilisation of p53 was only partially affected (50-70% of that in wild-type cells), suggesting that a Chk2-independent pathway for p53 stabilisation in response to IR exists (Takai *et al.*, 2002). Despite p53 protein stabilisation and phosphorylation of Ser23 (mouse equivalent of Ser20), p53 was completely transcriptionally inactive, and induction of target genes, such as p21, was not observed in *Chk2*<sup>-/-</sup> cells. These results suggest that while Chk2 plays a partially redundant role in p53 stabilisation, it is critical for p53 transcriptional activity in response to IR (Takai *et al.*, 2002).

Two recent reports question the role of Chk2 in the p53 DNA damage response (Ahn *et al.*, 2003; Jallepalli *et al.*, 2003). First, Ahn and colleagues used siRNA to downregulate Chk1, Chk2 or both Chk1 and Chk2 in human tumour cell lines. They observed that in cells where there was a marked reduction in Chk2, p53 is still Ser20 phosphorylated and stabilised, and activates target genes such as p21 after DNA damage with the radiomimetic compound NCS. In addition, a p53-derived peptide containing Ser20 proved a poor substrate for Chk2, and the sequence surrounding Ser20 does not fit the consensus Chk2 phosphorylation site found in other Chk2 substrates (O'Neill *et al.*, 2002). The authors conclude that Chk1 and Chk2 are unlikely to be regulators of p53.

Second, Jallepalli and colleagues disrupted the Chk2 gene in human cells through homologous recombination, and found that phosphorylation of p53 at Ser20, stabilisation of p53 protein, transcriptional activation of p53 target

genes, and cell cycle arrest and apoptosis were completely intact regardless of Chk2 status.

However, the most recent analysis of the Chk2-p53 link suggests that activation of Chk2 as a kinase towards Thr18 and Ser20 of p53 requires allosteric changes in Chk2 induced by its interaction with native full-length p53 (Craig *et al.*, 2003). Thus, two different classes of Chk2 substrates may exist, those similar to Cdc25 and others more similar to p53 (Bartek and Lukas, 2003). Phosphorylation of p53 at Thr18 by Chk2 has been reported to destabilise the p53/MDM2 complex (Craig *et al.*, 1999; Schon *et al.*, 2002), and it has been suggested that phosphorylation at Ser20 creates a phospho-consensus binding site for the transcriptional co-activator and histone acetyltransferase p300 (Dornan *et al.*, 2003). Thus, Chk2 may operate as a molecular switch, converting p53 from an MDM2 binding protein to a p300 binding protein subject to enhanced acetylation by p300 (Dornan *et al.*, 2003; Shimizu and Hupp, 2003). It is possible therefore, that in addition to regulating p53 stabilisation Chk2 regulates the activation of p53-dependent transcription. This is consistent with the observation that *Chk2*<sup>-/-</sup> mouse cells have a pronounced defect in p53-dependent transcription (Hirao *et al.*, 2000; Takai *et al.*, 2002).

In summary, the ability of p53 to activate cell cycle arrest in G1 phase through p21 in *Chk2*<sup>-/-</sup> deficient mice and MEFs is unclear, as is the role of Chk2 at both the S-phase and G2/M checkpoints. What is consistent is that *Chk2*<sup>-/-</sup> mice show increased resistance to IR, and that Chk2 regulates apoptotic processes (Hairo *et al.*, 2000; Jack *et al.*, 2002; Takai *et al.*, 2002). However, the resistance to apoptosis observed in *Chk2*<sup>-/-</sup> cells is likely attributable only in part to defects in p53 activation. A recent report has linked Chk2 and the

Promyelocytic Leukemia (PML) protein in the induction of p53-independent apoptosis (Yang *et al.*, 2002).

Interestingly, MDM2 is also phosphorylated by ATM after IR (Khosravi *et al.*, 1999). After IR, phosphorylation of Ser395 in MDM2 by ATM decreases the ability of MDM2 to shuttle p53 from the nucleus to the cytoplasm, thereby allowing the p53 protein to accumulate (Maya *et al.*, 2001). MDM2 can also be phosphorylated by Chk1 *in vitro*, although the sites phosphorylated have not yet been identified (Shieh *et al.*, 2000).

Therefore, in response to DNA damage, it is likely that ATM/ATR and Chk1/Chk2 contribute to the stabilisation and activation of p53 by modulating its interaction with proteins such as MDM2 and p300.

## 1.21 The S-phase checkpoint

One of the first abnormalities to be characterised in ATM-deficient cells was a failure to arrest DNA synthesis after IR (Houldsworth and Lavin, 1980; Painter and Young, 1980). This phenomenon was called radioresistant DNA synthesis (RDS). RDS is a measure of DNA synthesis soon after irradiation and it reflects cells that are already in S-phase, rather than cells entering S-phase from G1, and it does not depend on p53 (Larner *et al.*, 1994; Morgan *et al.*, 1994).

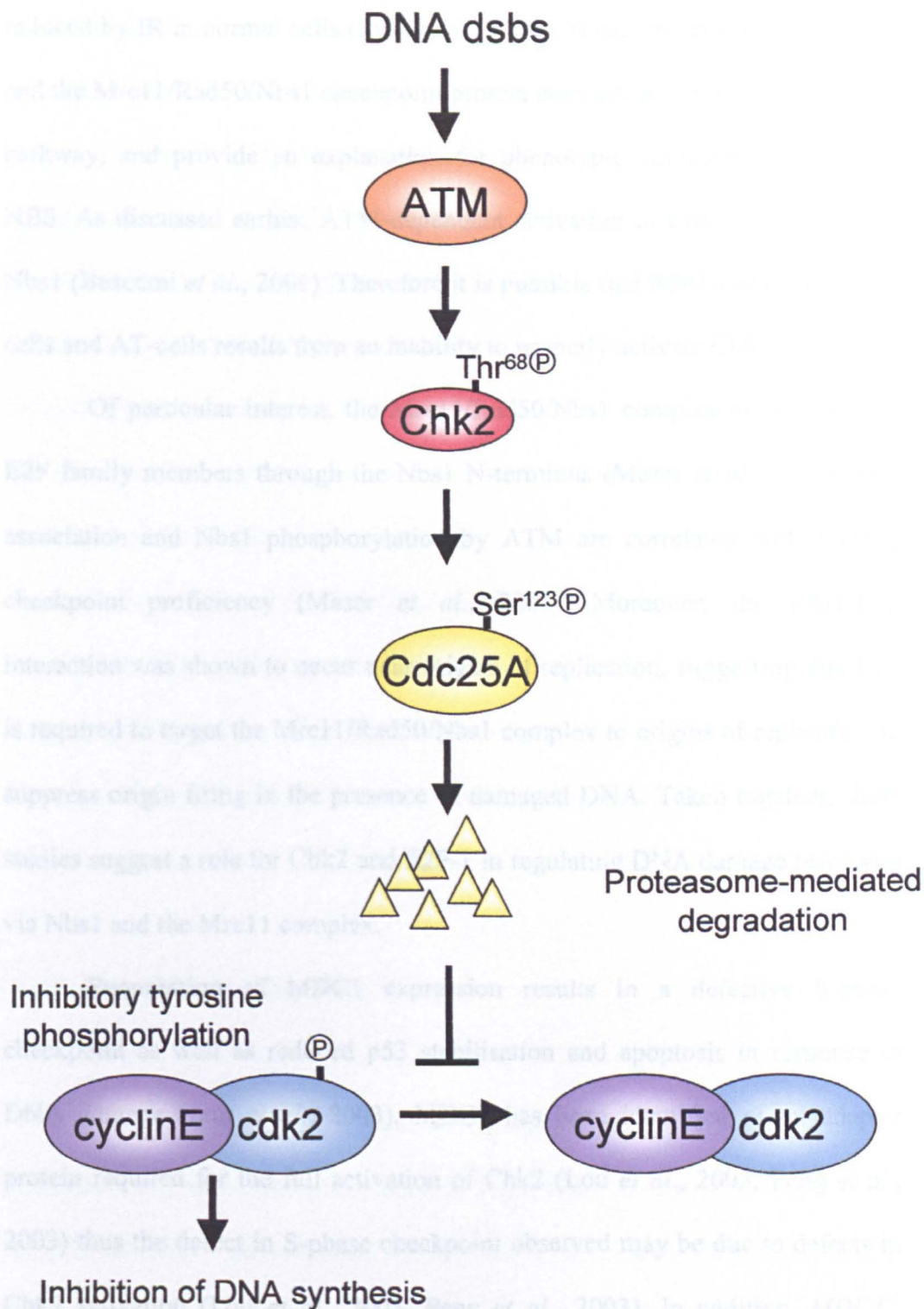
Human cells respond to UV-irradiation or IR by rapidly degrading the protein phosphatase Cdc25A, in a ubiquitin and proteasome-dependent manner (Mailand *et al.*, 2000). When active, Cdc25A removes phosphate groups from another enzyme, cdk2. This activates the cyclinE/cdk2 and cyclinA/cdk2

kinases, which are required for entry into and progression through S-phase (Mittnacht, 1998). Degradation of Cdc25A results in persistent tyrosine phosphorylation and inactivation of cdk2, and therefore inhibition of the cell cycle.

UV-irradiation causes a decrease in Cdc25A levels and suppression of DNA replication dependent on the enzyme Chk1 (Mailand *et al.*, 2000). IR also leads to degradation of Cdc25A, and Cdc25A is a substrate for Chk2 after IR (Falck *et al.*, 2001a). IR activates Chk2 in an ATM dependent manner, and activated Chk2 phosphorylates Cdc25A at Ser123 *in vitro* and on the same site *in vivo* in response to IR, and this modification targets Cdc25A for rapid ubiquitin-dependent, proteasome-mediated degradation (Falck *et al.*, 2001a; Figure 1.7). Furthermore, with the introduction of mutant Chk2 into a mammalian cell line, the downregulation of Cdc25A and S-phase delay that normally occurs in response to IR is blocked (Falck *et al.*, 2001a). Similarly, expression of a Cdc25A-resistant mutant of cdk2, or persistent expression of Cdc25A, blocks the IR induced S-phase checkpoint (Falck *et al.*, 2001a). The involvement of Chk2 in the S-phase checkpoint was recently confirmed when siRNA-mediated downregulation of Chk2 (or Chk1) resulted in a partial RDS phenotype (Sorenson *et al.*, 2003; Zhao *et al.*, 2002).

Importantly, the activation of Chk2, degradation of Cdc25A and inhibition of cdk2 are defective in AT-cells (Falck *et al.*, 2001a). NBS is a disorder that shares with AT a variety of phenotypic defects including radio-sensitivity and RDS (Carney, 1999). In response to IR, Nbs1 is phosphorylated on Ser343 in an ATM-dependent manner, and introduction of Nbs1 that has Ser343 mutated to alanine is sufficient to abrogate an S-phase checkpoint





**Figure 1.7 Role of ATM and Chk2 at the S-phase checkpoint**

In response to DNA double stranded breaks, ATM phosphorylates and activates Chk2. Phosphorylation of Cdc25A on Ser123 by Chk2 leads to the proteasome mediated degradation of Cdc25A and, in turn, the failure to dephosphorylate and activate cyclinE-cdk2 complexes which results in inhibition of DNA synthesis.

induced by IR in normal cells (Lim *et al.*, 2000). These observations link ATM and the Mre11/Rad50/Nbs1 checkpoint protein complex to a common signalling pathway, and provide an explanation for phenotypic similarities in AT and NBS. As discussed earlier, ATM-dependent activation of Chk2 by IR requires Nbs1 (Buscemi *et al.*, 2001). Therefore it is possible that RDS observed in NBS cells and AT-cells results from an inability to properly activate Chk2.

Of particular interest, the Mre11/Rad50/Nbs1 complex associates with E2F family members through the Nbs1 N-terminus (Maser *et al.*, 2001). This association and Nbs1 phosphorylation by ATM are correlated with S-phase checkpoint proficiency (Maser *et al.*, 2001). Moreover, the Nbs1-E2F interaction was shown to occur near origins of replication, suggesting that E2F is required to target the Mre11/Rad50/Nbs1 complex to origins of replication to suppress origin firing in the presence of damaged DNA. Taken together, these studies suggest a role for Chk2 and E2F-1 in regulating DNA damage responses via Nbs1 and the Mre11 complex.

Suppression of MDC1 expression results in a defective S-phase checkpoint as well as reduced p53 stabilisation and apoptosis in response to DNA damage (Lou *et al.*, 2003). MDC1 has been identified as an adapter protein required for the full activation of Chk2 (Lou *et al.*, 2003; Peng *et al.*, 2003) thus the defect in S-phase checkpoint observed may be due to defects in Chk2 activation (Lou *et al.*, 2003; Peng *et al.*, 2003). In addition, *MDC1*<sup>-/-</sup> deficient cells fail to activate the intra-S-phase checkpoint (in addition to the G2/M phase checkpoint) properly in response to IR, however this was associated with an inability to regulate Chk1 properly (Stewart *et al.*, 2003).

Finally, MDC1 is phosphorylated in an ATM dependent manner in response to IR, which leads to its rapid relocalisation to nuclear foci that also contain the Mre11/Rad50/Nbs1 complex, H2AX and 53BP1 (Goldberg *et al.*, 2003). Suppression of MDC1 expression prevents IR induced foci formation by the Mre11/Rad50/Nbs1 complex, and this event is attributed with causing an RDS phenotype. Interestingly, down-regulation of MDC1 does not affect IR induced phosphorylation of Chk2 at Thr68 or activation of degradation of Cdc25A, suggesting that these events may be involved in, but are not critical for the intra-S-phase checkpoint response.

In summary, the idea that Chk2 regulates the intra-S-phase checkpoint in response to IR via the targeted degradation of Cdc25A carries some weight. However more studies are needed to clarify the role of proteins such as the recently identified MDC1 before one can be sure of the molecular mechanisms that are involved.

## **1.22 The G2 checkpoint**

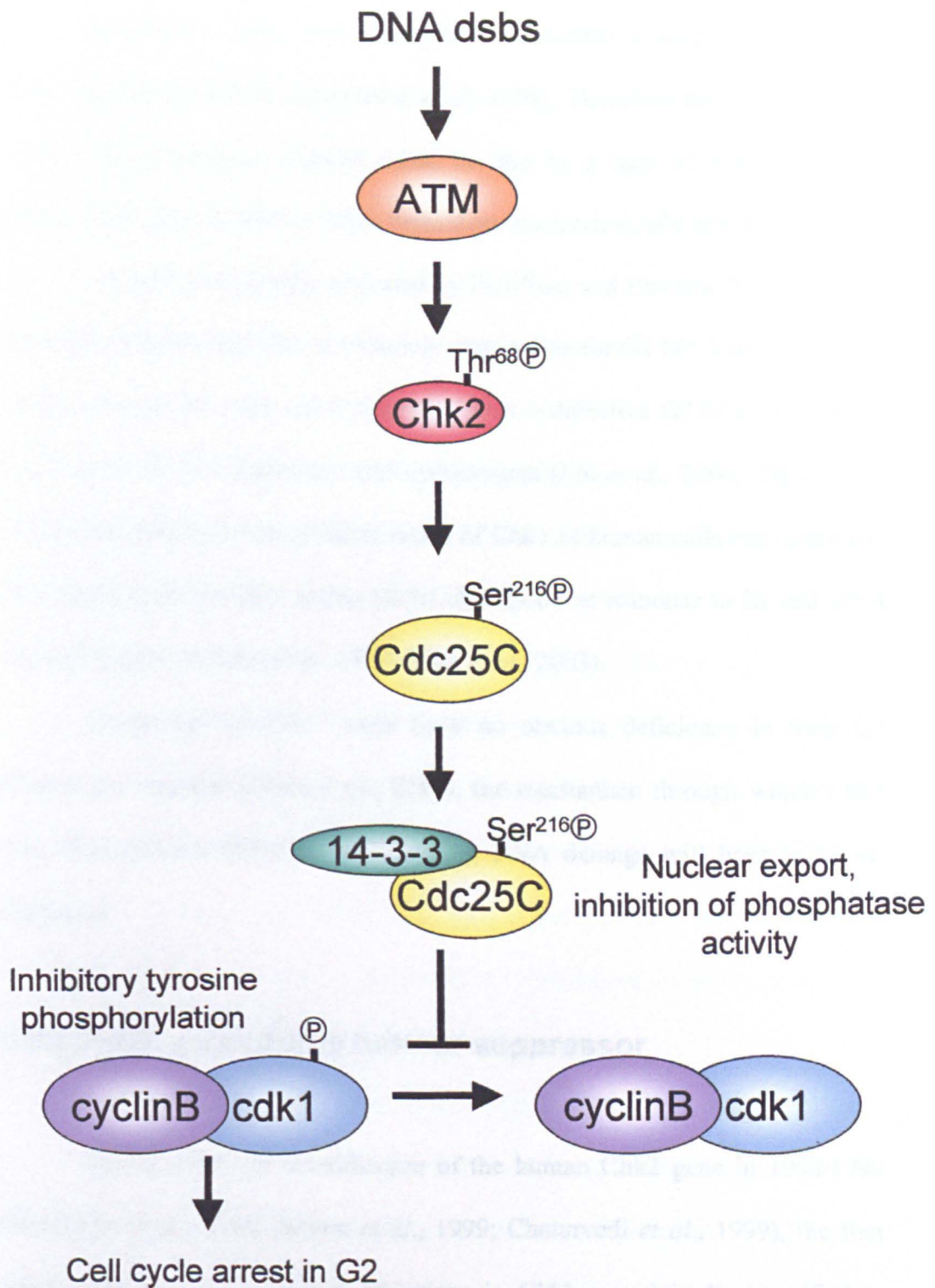
In mammalian cells, G2 arrest is regulated mainly by the maintenance of an inhibitory tyrosine phosphorylation of cdk1. Cdk1 activation is catalysed by the dual specificity phosphatase Cdc25C, which removes the inhibitory tyrosine phosphorylation required to allow cells to progress from G2 into mitosis (Morgan, 1997).

Arrest in G2 begins with phosphorylation of Cdc25C on Ser216 (Sanchez *et al.*, 1997; Peng *et al.*, 1997). This phosphorylation both inhibits the phosphatase activity of Cdc25C and contributes to its cytoplasmic sequestration

by an interaction with 14-3-3 proteins (Peng *et al.*, 1997), which are a family of ubiquitous phospho-binding proteins. Because it is trapped in the cytoplasm, Cdc25C cannot perform its nuclear function, which is to dephosphorylate and activate cdk1.

Both Chk2 and Chk1 phosphorylate Cdc25C at Ser216 *in vitro* (Peng *et al.*, 1997), and this site is phosphorylated to high stoichiometry *in vivo* (Peng *et al.*, 1997; Sanchez *et al.*, 1997). A model for the G2 checkpoint proposes that Chk2 or Chk1 act by phosphorylating Ser216 to maintain Cdc25C in its inhibitory phosphorylated state until DNA damage is repaired (Figure 1.8). However, expression of Cdc25C with a non-phosphorylatable alanine residue at Ser216 has only a modest effect on the G2 damage checkpoint (Peng *et al.*, 1997). Furthermore, 14-3-3-dependent regulation of Cdc25C localisation is neither necessary nor sufficient for checkpoint regulation (Lopez-Girona *et al.*, 2001; Graves *et al.*, 2001). Therefore, phosphorylation of Ser216 is unlikely to be the only event preventing the activation of cdk1 in the presence of DNA damage.

Genetic studies partially support the involvement of Chk2 in the control of the G2/M checkpoint. Embryonic stem (ES) cells that lack Chk2 cannot maintain an IR induced G2 arrest properly (Hairo *et al.*, 2000). However they do arrest for up to 12 hours following IR suggesting that although Chk2 is not required for the initial arrest, it is required for maintenance of the arrest (Hairo *et al.*, 2000). Contrary to the results obtained by Hairo and colleagues, MEFs isolated from a second, independently derived *Chk2*<sup>-/-</sup> mouse showed no defects in G2/M checkpoints (Takai *et al.*, 2002).



**Figure 1.8 Role of ATM and Chk2 at the G2 checkpoint**

In response to DNA double stranded breaks, ATM phosphorylates and activates Chk2. Phosphorylation of Cdc25C on Ser216 by Chk2 leads to the formation of 14-3-3-Cdc25C complexes. 14-3-3 inhibits the phosphatase activity of Cdc25C, and sequesters it to the cytoplasm where it is unable to carry out its normal function. The failure of Cdc25C to dephosphorylate and activate cyclinB-cdk1 complexes results in arrest of the cell cycle in G2 phase.

In somatic cells, p53 is required to sustain a long-term G2 arrest following IR-induced damage (Bunz *et al.*, 1998). Therefore the failure of *Chk2*<sup>-/-</sup> ES cells to remain arrested could be due to a lack of Chk2-dependent activation of p53, as well as Chk2-dependent inactivation of Cdc25C.

Despite being poorly activated by IR (Zhao and Piwnica-Worms, 2001), evidence suggest that Chk1 is primarily responsible for G2 DNA damage arrest. Chk1 deficient ES cells and blastocysts show a defective G2/M checkpoint in response to IR, UV-irradiation and hydroxyurea (Liu *et al.*, 2000; Takai *et al.*, 2000), and siRNA-mediated knockdown of Chk1 in human cells has confirmed the requirement for Chk1 in the G2/M checkpoint in response to IR and DNA damaging agents (Gatei *et al.*, 2003; Xiao *et al.*, 2003).

Given that *Cdc25C*<sup>-/-</sup> mice have no obvious deficiency in their G2 checkpoint response (Chen *et al.*, 2001), the mechanism through which Chk1 and Chk2 enforce G2/M arrest following DNA damage will have to be re-evaluated.

## **1.23 Chk2, a candidate tumour suppressor**

Shortly after the identification of the human Chk2 gene in 1998/1999 (Matsuoka *et al.*, 1998; Brown *et al.*, 1999; Chaturvedi *et al.*, 1999), the first *Chk2* mutations were reported. Mutations in *Chk2* were initially identified in some cases of LFS (Bell *et al.*, 1999; Lee *et al.*, 2001b; Vahteristo *et al.*, 2001), a hereditary cancer susceptibility usually associated with inherited mutations in the *TP53* gene. These findings suggested that germline mutations of Chk2 could account for the subset of LFS families who do not have mutation in p53.

Sporadic mutations of Chk2 have been identified in a subset of diverse human malignancies including breast cancers, lung cancers, colon cancer, osteosarcomas and lymphoid tumours (Bell *et al.*, 1999; Sullivan *et al.*, 2002; Miller *et al.*, 2002; Matsuoka *et al.*, 2001; Haruki *et al.*, 2000; Hofmann *et al.*, 2001).

The majority are heterozygous mutations that lead to the generation of a truncated protein, or are missense mutations in important domains such as the SCD, kinase and FHA domain. Biochemical analysis confirmed that mutants within the FHA domain have lost their ability to interact with, and efficiently phosphorylate substrates such as Cdc25A, p53 or BRCA1 (Li *et al.*, 2002; Falck *et al.*, 2001a; Falck *et al.*, 2001b; Wu *et al.*, 2001b), while mutants within the kinase domain show decreased or lost kinase activity (Matsuoka *et al.*, 2001; Wu *et al.*, 2001b). Some of the FHA domain or kinase domain mutants are unstable proteins and are therefore expressed at much lower levels than wild-type Chk2 (Matsuoka *et al.*, 2001; Lee *et al.*, 2001b). Other mutants function in a dominant-negative manner by disrupting the function of the endogenous wild-type protein (Falck *et al.*, 2001a).

Therefore, mutations in *Chk2* could lead to partial loss of function (one allele results in a truncated inactive protein), or the generation of a protein that acts in a dominant-negative fashion, or both simultaneously. The loss of function nature of some tumour associated Chk2 mutations support its candidacy as a tumour suppressor.

Most interestingly, concomitant loss of function mutations in Chk2 and its substrate p53 were identified in the sporadic colon carcinoma cell line HCT-15, which has one allele mutated in the FHA domain, and one normal allele (Bell *et*

*al.*, 1999; Falck *et al.*, 2001b). It is possible that this combination of mutations might provide an additional selective advantage to tumour cells, compared to cells that have defects in Chk2 or p53 alone (Falck *et al.*, 2001b).

Intriguingly, constitutively active (Thr68 phosphorylated) Chk2 has been found in human cancer cell lines, and human primary breast and colon carcinomas (DiTullio *et al.*, 2002). Persistent activation of Chk2 likely increases the selective pressure in human cancers to mutate p53 (DiTullio *et al.*, 2002).

A subset of tumours have been identified that have undetectable Chk2 protein, in the absence of any mutations in the Chk2 gene (Bartkova *et al.*, 2001; Vahteristo *et al.*, 2002; Sullivan *et al.*, 2002; Tort *et al.*, 2002). Epigenetic mechanisms are also known to undermine the expression of some tumour suppressors, however silencing of gene expression through promoter methylation has been excluded as a cause of this phenotype (Sullivan *et al.*, 2002; Tort *et al.*, 2002). The finding of normal mRNA levels of Chk2 in such cases suggests that post-transcriptional aberrations of Chk2 downregulate Chk2 levels during oncogenesis (Bartek and Lukas, 2003).



## 1.24 Objectives

It is well established that E2F transcription factors play a central role in co-ordinating cell cycle progression at the G1 to S-phase transition (Dyson, 1998). In addition to regulating proliferation, E2F controls the expression of genes involved in apoptosis. Whilst this study was underway, recent studies have shown that E2F-1 is regulated in response to DNA damage, as treatment of cells with DNA damaging agents such as those used in cancer chemotherapy increases E2F-1 protein levels (Blattner *et al.*, 1999; O'Conner *et al.*, 2000). Although little is known about the mechanism of E2F-1 induction, it has been suggested that ATM may be involved in the phosphorylation and stabilisation of E2F-1 in response to DNA damage (Lin *et al.*, 2001).

The aim of this study was to further explore the pathways that signal DNA damage to E2F-1, and gain insight into the role of E2F-1 as an effector protein in the cellular response to DNA damage. More specifically the aim was to:

- Explore the induction of E2F-1 in response to DNA damage
- Identify the signal transduction pathways responsible for the regulation of E2F in response to DNA damage
- Investigate the mechanism involved in E2F-1 induction
- Address the physiological consequence of E2F-1 induction and activation

## Chapter 2. Materials and Methods

### 2.1. Plasmids and site-directed mutagenesis

The following plasmids have been previously described: pCMV- $\beta$ -gal and pCMV-CD20 (Allen *et al.*, 1997), pCMV-DP1 (Bandara *et al.*, 1993), pcyclinE-luciferase (Morris *et al.*, 2000), Apaf-1-luciferase (–396/+208; Moroni *et al.*, 2001) and Exon1- $\beta$ -luciferase (Bates *et al.*, 1998). pCMV-HA-E2F-1 wild-type is as described (Helin *et al.*, 1992), and was used as a template to construct pCMV-HA-S364A using the *In vitro* mutagenesis system Quickchange (Stratagene) as recommended by the manufacturer. The primers used were (bases which differ from wild-type E2F-1 are underlined):

Fwd 5'-TTGTCCCGGATGGGCGCCCCTGCGGGCTCCC- 3'

Rev 3'-AACAGGGCCTACCCGCGGGACGCCCCGAGGG- 5'

For expression in *E.coli*, pCMV-HA-E2F1 wild-type and S364A were digested with BamHI and SacI and the resulting insert was cloned into pGEX<sup>KG</sup> (Pharmacia) at the same sites. 6-His-tagged Chk2 wild-type, Flag-Chk2 wild-type and Flag-Chk2-D347A were gifts from T. Halazonetis (Chehab *et al.*, 2000). Baculovirus vectors for His-cyclinA and His-cdk2 were as described (Desai *et al.*, 1995).

## **2.2. GST and His-tagged recombinant protein purification**

Transformed BL21 bacteria (Invitrogen) were grown to mid-logarithmic phase in 500ml of LB-broth containing the appropriate antibiotic at 37°C. Thereafter, protein expression was induced by the addition of 0.5mM (final concentration) isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) for 4 h at 30°C.

For GST purification, bacterial pellets were resuspended in 10ml PBS/1% Triton X-100 and 0.5mM PMSF on ice, and then sonicated briefly (3x10 sec) on ice. Bacterial debris was pelleted by centrifugation at 10,000rpm for 20 min at 4°C. A 400 $\mu$ l suspension of glutathione-Sepharose beads (50% v/v; Amersham), that had been pre-washed in PBS, was added to the supernatant and mixed with constant rotation at 4°C for 30 min. The suspension was washed three times with 50ml PBS by spinning in a bench-top centrifuge at 5,000rpm for 5 min at 4°C. The GST proteins were eluted from the beads by incubating the bead pellet with an equal volume of 50mM Tris pH8, containing 10mM of glutathione.

For His-tagged purification, the same procedure was followed as for GST purification except; a 400 $\mu$ l suspension of Ni-NTA nickel-agarose beads (50% v/v, Qiagen) was used to bind His-tagged proteins, and His-tagged proteins were eluted by incubating the beads with an equal volume of imidazole buffer (200mM imidazole, 100mM NaCl and 20mM Tris-HCL pH8). To remove imidazole, purified proteins were dialysed with buffer containing (100mM NaCl and 20mM Tris-HCL pH8), overnight at 4°C.

Purified Cdc25C protein was purchased from Biodiagnostics.

## **2.3. Protein purification from baculovirus infected Sf9 cells**

*Sf9* insect cells were co-infected with the appropriate recombinant baculoviruses and whole cell extracts were prepared 48 h post-infection. Briefly, cells were lysed in buffer A (25mM HEPES pH7.6, 400mM KCL, 12.5mM MgCl<sub>2</sub>, 0.1mM EDTA, 30% glycerol (v/v), 0.1% NP40 (v/v)), and then centrifuged to remove insoluble material. The resulting lysate was diluted dropwise with 2 volumes of buffer B (25mM HEPES pH7.6, 100mM KCL, 12.5mM MgCl<sub>2</sub>, 0.1mM EDTA, 10% glycerol (v/v), 0.1% NP40 (v/v)), and then incubated with Ni-NTA beads (Qiagen) for 1 h at 4°C. The beads were washed three times with buffer B containing 200mM KCL and 20mM imidazole. His-tagged proteins were eluted with 250mM imidazole in buffer B and dialysed against the same buffer without the imidazole.

## **2.4. Cell culture**

MCF7 (human mammary epithelial), U2OS (human osteosarcoma epithelial), T98G (human glioblastoma fibroblasts), and SAOS2 (human osteosarcoma epithelial) tumour cell lines were grown in Dulbecco's modified eagles medium (DMEM, Gibco) supplemented with 10% FCS (Gibco) and antibiotics, 10mg/ml streptomycin and 100U/ml penicillin (Gibco) at 37°C in a 5% CO<sub>2</sub>/H<sub>2</sub>O-saturated atmosphere. HCT-15 (human colon carcinoma) cells were grown in RPMI-40 (Gibco) supplemented with 10% FCS. AT (human fibroblast) cells, repository number GM02530 were purchased from the Coriell

Institute for Medical Research (New Jersey, USA) and were grown in DMEM supplemented with 10% FCS. *Sf9* (*Spodoptera frugiperda* insect cells) were grown in TC100 (Gibco) supplemented with 5% FCS.

## 2.5. Transient Transfection

Cells for transient transfection were plated out 24 h before transfection at approximately  $1.5 \times 10^6$  cells per 100mm dish (for immunoprecipitation and immunoblotting),  $5 \times 10^5$  cells per 60mm dish (for luciferase and  $\beta$ -galactosidase assays, and gel retardation) or  $2 \times 10^4$  cells per 35mm dish (on coverslips for immunofluorescence). For calcium phosphate precipitation, the relevant plasmid DNA was mixed with a final concentration of 250mM  $\text{CaCl}_2$  and the resulting solution (100 $\mu$ l per 35mm dish, 250 $\mu$ l per 60mm dish and 500 $\mu$ l per 100mm dish) was added dropwise to an equal volume of 2xHBS solution (50mM HEPES pH 7.1, 280mM NaCl, and 1.5mM  $\text{Na}_2\text{HPO}_4$ ), and incubated at room temperature for 30 min before addition to the media on cells. For Lipofectamine transfection (Gibco), 2 $\mu$ l of Lipofectamine was used for every 1 $\mu$ g of DNA transfected. To maintain a constant amount of plasmid DNA in each sample, pCDNA3 (Invitrogen) was transfected as appropriate. Cells were washed three times with PBS at 14-16 h post-transfection. Cells were harvested after a further incubation of 24 h.

## **2.6. Cell extract preparation**

Cells were lysed in ice-cold extraction buffer (50mM Tris (pH 7.4), 150mM NaCl<sub>2</sub>, 5mM EDTA, 0.5% NP-40, 1mM PMSF, 5mM NaF, 1mM sodium vanadate, 1 x Protease Inhibitor Cocktail) for 30 min and centrifuged for 10 min to remove insoluble material. The protein content of cell extracts was measured using Biorad reagent (Biorad).

## **2.7. Immunoblotting**

Typically, 50-100µg of cell extract was immunoblotted depending on whether the protein to be detected was transfected or endogenous. Samples were resolved by denaturing gel electrophoresis (typically 10% gels) and electro-transferred to Protran nitrocellulose membrane (Inverclyde Biochemicals Ltd), blocked in PBS-10% non-fat milk for 30 min, then incubated with primary antibody overnight at 4°C in PBS-5% non-fat milk-0.1% Tween-20. After washing (3x10min) in PBS-Tween-20, the blot was incubated with secondary antibody, either anti-rabbit or anti-mouse alkaline phosphatase conjugated antibody (Promega; 1:5000), or horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Amersham; 1:5000), for 1 h at room temperature in PBS-5% non-fat milk-0.1% Tween-20. After washing (3x10min) in PBS-Tween-20, proteins were visualised by incubation with alkaline phosphatase substrate (Sigma) or ECL reagent (Pierce). Equal protein loading was confirmed with Ponceau S staining.

## 2.8. Antibodies

E2F-1 antibody (KH-95), E2F-2 antibody (C-20), E2F-3 antibody (N-20), PCNA antibody (PC-10), p53 antibody (DO-1), and PML antibody (PG-M3) were purchased from Santa Cruz. Apaf-1 antibody (NT), and p73 antibody (GC-15) were purchased from Upstate Biotechnology. p19<sup>ARF</sup> antibody (Ab-2) was purchased from Neomarkers. Flag M2 antibody was purchased from Sigma. HA-11 antibody was purchased from Cambridge Bioscience. Chk2 phosphorylated at Thr68 antibody was purchased from Cell Signalling Technology. Anti-P-S364 rabbit polyclonal antibody was generated by Eurogentec, Belgium. Briefly, two peptides were generated based on the sequence around Ser364, one of which was chemically phosphorylated at Ser364. Peptides were conjugated to KLH carrier protein, and the phosphorylated peptide was used for immunisation. Antibodies were affinity purified over a column containing the immobilised phosphorylated peptide. Then the bound material was passed over a second column containing immobilised non-phosphorylated peptide. P-S364 specific antibodies appeared in the flow-through of the second peptide column (see figure 4.4a).

## 2.9. Immunoprecipitation

For immunoprecipitation of proteins, the relevant antibodies were incubated with 30µl of Protein A beads (Sigma) overnight at 4°C with rotation, together with 500µl cell extract (1-2mg) prepared in extraction buffer (50mM Tris (pH 7.4), 150mM NaCl<sub>2</sub>, 5mM EDTA, 0.5% NP-40, 1mM PMSF, 5mM

NaF, 1mM sodium vanadate, 1 x Protease Inhibitor Cocktail). The bead pellets were then washed 5 times in lysis buffer before being resuspended in 1x SDS-loading buffer (250mM Tris-HCL pH 6.8, 20% glycerol (v/v), 4% SDS (w/v), 0.1% bromophenol blue (w/v), 200mM DTT, and 5%  $\beta$ -mercaptoethanol (v/v)) and analysed by denaturing gel electrophoresis and immunoblotting. For *in vitro* immunoprecipitation of cold kinase assays, 40 $\mu$ l of protein A beads were resuspended in 400 $\mu$ l PBS together with 1 $\mu$ l of antibody, and incubated at 4°C for 4 h with rotation. Beads were then centrifuged and buffer removed. Kinase assay reaction products (30 $\mu$ l) were made to 400 $\mu$ l with PBS and added to protein A beads with antibody bound, and incubated overnight at 4°C with rotation. The bead pellets were then washed 3 times in PBS before being resuspended in 1x SDS-loading buffer and analysed by denaturing gel electrophoresis and immunoblotting.

## **2.10. *In vitro* protein expression**

*In vitro* transcription and translation was carried out using a TNT T7 coupled reticulocyte lysate system (Promega) as recommended by the manufacturer, in the presence of  $^{35}$ S methionine. For expression of unlabelled protein, reactions were supplemented with methionine. To check expression 1 $\mu$ l was subjected to denaturing gel electrophoresis and autoradiographed or immunoblotted.



## 2.11. *In vitro* binding assays

For GST-pull down assay proteins were *in vitro* transcribed and translated to incorporate  $^{35}\text{S}$ -methionine using the T7 TNT coupled reticulocyte lysate system (Promega) as described by the manufacturer. An equal amount of  $^{35}\text{S}$ -labelled *in vitro* translated protein was added to 1 $\mu\text{g}$  GST, GST-E2F-1 or GST-S364A protein in 200 $\mu\text{l}$  TNE buffer (1mM DTT, 1mM PMSF, and 1x protease inhibitor cocktail (Sigma), and incubated overnight with rotation at 4°C. Beads were then washed 3 times in TNE buffer and bound proteins eluted in 30 $\mu\text{l}$  of 1x SDS loading buffer and visualised by gel electrophoresis and autoradiography (Biorad).

## 2.12. *In vitro* kinase assays

Purified recombinant wild-type or mutant E2F-1 proteins (1 $\mu\text{g}$ ), and Cdc25C (1 $\mu\text{g}$ ) were incubated with purified His-tagged Chk2 protein (0.2 $\mu\text{g}$ ) for 30 min in 1x kinase buffer (50mM HEPES pH7.4, 10mM  $\text{MgCl}_2$ , 10mM  $\text{MnCl}_2$ , 10mM DTT) supplemented with 50 $\mu\text{M}$  unlabeled ATP and 5 $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ] ATP. Reactions to be used for immunoblotting with P-S364 antibody were performed cold with 1mM ATP without [ $\gamma$ - $^{32}\text{P}$ ].

Purified recombinant wild-type or mutant E2F-1 proteins (1 $\mu\text{g}$ ), and pRb (1 $\mu\text{g}$ ) were incubated with baculovirus purified cyclinA/cdk2 (0.2 $\mu\text{g}$ ) for 30 min in 1x kinase buffer (20 mM HEPES (pH 7.6), 10mM  $\text{MgCl}_2$ , 1mM EDTA,

2mM MnCl<sub>2</sub>, 10% glycerol, 1mM DTT) supplemented with 50μM unlabeled ATP and 5μCi [γ-<sup>32</sup>P] ATP.

Reactions were stopped by the addition of 3x SDS loading buffer and the protein mixtures were resolved by denaturing gel electrophoresis and subjected to autoradiography to monitor γ-<sup>32</sup>P incorporation, or immunoblotted with antibody anti-P-S364 to monitor phosphorylation of E2F-1 on Ser364.

## 2.13. Gel Retardation analysis

The E2F recognition site from the adenovirus E2A promoter (or a mutant site) was used in all gel retardation analysis (Girling et al., 1993). The E2F binding site is in bold, and the nucleotides changed in the mutant site are underlined.

Wild-type    5'-GATCTAGTTTTCGCGCTTAAATTTGA-3'  
                  3'-ATCAAAAGCGCGAATTAAACTCTAG-5'

Mutant        5'-GATCTAGTTTTCGATATTAAATTTGA-3'  
                  3'-ATCAAAAGCTATAAATTAAACTCTAG-5'

Typically, 10μg of cell extract prepared in lysis buffer (50mM KCL, 50mM Tris pH7.6, 25% glycerol, 2mM DTT, 0.1% Triton X-100, 0.2mM PMSF, and 1x protease inhibitor cocktail (Sigma)) was used per gel retardation sample. The cell extract was combined with binding buffer (10mM HEPES pH7.6, 100mM KCL, 1mM EDTA, 4% Ficoll, 0.5mM DTT), 2μg of sheared salmon sperm DNA and 200ng of mutant promoter oligonucleotide to reduce the non-specific

DNA binding activity. For gel retardation using recombinant and *in vitro* translated proteins, proteins were combined with binding buffer, 2µg of sheared salmon sperm DNA and 200ng of mutant promoter oligonucleotide to reduce the non-specific DNA binding activity.

Antibodies for supershifts were added and complexes were allowed to form at room temperature. After 15 min, 1ng of a <sup>32</sup>P-labelled E2F site was added for a further 20 min. Complexes were resolved on a 4% polyacrylamide gel in 0.5x Tris-borate EDTA (TBE) at 4°C for 2 h (200V).

## **2.14. Reporter gene assays**

For reporter gene assays U2OS cells were transfected with the indicated amount of plasmid DNA together with 500ng of CMV-β-gal as a control for transfection efficiency. The total amount of DNA transfected was kept constant by the addition of pCDNA3. Transfected cells were washed twice in PBS and regrown in DMEM-FCS for a further 24 h. Cells were then harvested, or treated with etoposide for the indicated time before harvesting in 300µl 1x reporter lysis buffer (25mM Tris-H<sub>3</sub>PO<sub>4</sub> pH 7.8, 2mM 2,2-diaminocyclohexane tetra acetic acid, 2mM DTT, 10% glycerol and 1% triton (Promega). Cell lysate was then micro-centrifuged at 10,000 rpm for 5 min to remove cell debris.

For the luciferase assay, 60µl of cell lysate was mixed with 300µl of luciferase reagent (Promega) and measured on a luminometer (Berthold).

For the  $\beta$ -galactosidase assay, 100 $\mu$ l of cell lysate was incubated with 100 $\mu$ l of 2x  $\beta$ -gal reagent (200mM sodium phosphate buffer pH 7.2, 2mM magnesium chloride, 100mM  $\beta$ -mercaptoethanol, 1.3 $\mu$ g/ml ONPG) and incubated at 37°C until a yellow colour developed. Reactions were stopped by the addition of 600 $\mu$ l H<sub>2</sub>O and the activity was measured in a spectrophotometer (Pharmacia) at a wavelength of 420nm.

## **2.15. DNA damaging agents**

Cisplatin, etoposide and doxorubicin were obtained from Sigma and were dissolved in DMSO. Cells were treated with drugs as indicated in the results section. For treatment, drugs were diluted to the appropriate concentration in fresh media before being added to cells. For UV-irradiation, cells were washed in PBS, and then placed in a UV crosslinker (Amersham) with dish lid removed, and irradiated with UV-light at 50 J/m<sup>2</sup>. Cells were then replenished with fresh media.

## **2.16. Cycloheximide treatment**

To ensure equal transfection efficiency among samples for cycloheximide treatment, large plates were transfected with HA-E2F1 or HA-S364A (10 $\mu$ g) in combination with CMV-DP1 (10 $\mu$ g) and pCDNA3 (20 $\mu$ g). After 16 h of incubation, transfected cells were washed 3 times with PBS before treatment with trypsin (Gibco) to release cells from the plates. Cells were then thoroughly

resuspended in 80ml of DMEM-FCS before re-plating onto eight 100cm plates. After a further 24 h of incubation, cells were either left untreated or treated with etoposide (10 $\mu$ M) for 4 h. Cycloheximide (50 $\mu$ g) was then added and cell extracts prepared and resolved for HA-E2F-1 or HA-S364A at the indicated times by immunoblotting with an HA-specific antibody.

## **2.17. Immunofluorescence**

For immunofluorescence, cells were seeded on 13mm borosilicate glass coverslips at a density of  $2 \times 10^4$  cells. 24 h later cells were treated with 10 $\mu$ M etoposide or 50J/m<sup>2</sup> UV-irradiation where indicated. 16 h post etoposide treatment cells were washed with PBS two times, fixed with 4% paraformaldehyde at room temperature, washed in PBS, then permeabilized in PBS containing Triton X-100 (0.1%) for 10 min. Fixed cells were then washed three times with PBS and blocked with PBS containing 10% FCS for 30 min. Cells were then incubated in primary antibody diluted in PBS-1% FCS for 30 min. Subsequently the coverslips were washed in PBS-1% FCS, before incubation with secondary antibody diluted in PBS-10% FCS for 30 min. After 3 final washes in PBS, the coverslips were mounted onto slides using citifluor (Citifluor Ltd).

Affinity purified anti-PS364 polyclonal antibody was used to detect endogenous phosphorylated E2F-1. For peptide competition antibody and peptide were pre-incubated for 10 min at room temperature. Secondary anti-rabbit and anti-mouse Alexa 594 antibodies were from Molecular probes. Cells were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI). Images

were captured using an Olympus BX60 system microscope with Hamamatsu C4742-95 digital camera. Images were analysed using Improvision Openlab digital imaging software.

## **2.18. Flow cytometry**

$1 \times 10^6$  U2OS cells seeded in a 10cm dish were transfected with the indicated amounts of expression vectors together with 8 $\mu$ g of pCMV-CD20 DNA. 48 h post-transfection cells were exposed to 20 $\mu$ M etoposide. Cells were harvested 12 h after exposure to etoposide in PBS, incubated with CD20 FITC conjugated antibody for 40 min, and fixed in 50% (v/v) ethanol/PBS. Cells were treated with Rnase (125U/ml) for 30 min before re-suspension in propidium iodide (20 $\mu$ g/ml) in PBS at 4°C for 1 h. Cells were analysed in a Becton Dickinson FACScan using cell-quest software. 10,000 CD20-stained cells were counted in each case

For SAOS2 cells,  $8 \times 10^5$  cells were transfected as described above and 65 h post-transfection exposed to 20 $\mu$ M etoposide and harvested 6 h later.

## **2.19. Denaturing gel electrophoresis**

Denaturing gels throughout refers to SDS-page.

# Chapter 3. E2F-1 is regulated by the DNA damage signalling pathway

## 3.1. Introduction

DNA damaging agents such as UV-irradiation, ionising radiation, or chemotherapeutic drugs can cause cell cycle arrest as well as apoptosis, largely dependent on p53. Several recent reports have described an increase in E2F-1 protein levels following the treatment of a variety of tumour cells with DNA damaging agents (Blattner *et al.*, 1999; O'connor *et al.*, 2000; Lin *et al.*, 2001). Little information is available on the mechanisms involved in the stress induction of E2F-1, although a recent study suggested that ATM phosphorylates and stabilises E2F-1, perhaps through a mechanism that involves the ubiquitin ligase p45<sup>SKP2</sup> (Lin *et al.*, 2001), or the BRCT-domain containing protein TopBP1 (Liu *et al.*, 2003).

The DNA damage signalling pathway is a highly conserved response to genotoxic stress (Durocher and Jackson, 2001). Central to the DNA damage checkpoint are ATM and ATR, which are believed to transduce signals to downstream targets, such as the checkpoint kinases Chk1 and Chk2 (Durocher and Jackson, 2001). In this respect, whilst ATM and ATR are generally regarded as sensors of DNA damage, the checkpoint kinases are likely to act as effectors of the response through the phosphorylation of key substrates, such as p53, the cell cycle-regulating phosphatases Cdc25A and Cdc25C, BRCA1, and PML (Bartek and Lukas, 2003).

In this chapter the role of the DNA damage signalling pathway, specifically Chk2, in the regulation of E2F-1 induction has been explored.

## **Results**

### **3.2. Induction of E2F-1 in tumour cells following DNA damage**

To investigate the regulation of endogenous E2F-1 in response to cellular stress, various tumour cell lines were treated with the chemotherapeutic drugs etoposide (complexes with topoisomerase II and DNA to enhance double-strand and single-strand cleavage of DNA), cisplatin (forms adducts with the DNA dinucleotide d(pGpG), inducing intrastrand crosslinks) or doxorubicin (intercalates in DNA), as well as UV-irradiation. Four cell lines were selected which each differed in their status of p53 and pRb. Western blot analysis of cell extracts revealed the degree of induction depends on the cell line and the drug being used, suggesting that the signalling pathways that are activated may vary. However, E2F-1 is clearly induced in each of these cell lines (Figure 3.1). These results are consistent with recent reports that E2F-1 is induced in various cell lines in response to a variety of forms of DNA damage including IR, UV-irradiation, and chemotherapeutic drugs (Blattner *et al.*, 1999; O'connor *et al.*, 2000; Lin *et al.*, 2001). These results also suggest that while different pathways may be involved in signalling to E2F-1, the induction of E2F-1 is a general response to DNA damage. The observation that E2F-1 is induced in SAOS2



cells argue strongly that the accumulation of E2F-1 in response to DNA damage can occur independently of p53 and pRb.

### **3.3. Specific induction of E2F-1 following DNA damage**

The accumulation of E2F-1 is regulated during the cell cycle, with mRNA and protein levels peaking at the G1 to S phase transition (Dyson, 1998). E2F-1, E2F-2 and E2F-3 family members are commonly regulated with respect to the cell cycle, whereas E2F-4 and E2F-5 are uniformly expressed throughout the cell cycle (Dyson, 1998). In response to DNA damage the pRb protein is hypo-phosphorylated (Ameltem *et al.*, 1996; Krtolica *et al.*, 1998), likely through the activation of cdis such as p21. Therefore the accumulation of E2F-1 observed in response to DNA damage could simply reflect the accumulation of cells arrested by pRb in the G1 phase, when E2F-1 levels are naturally high. If this was the case, then the protein levels of similarly regulated family members such as E2F-2 and E2F-3 would also be expected to increase. To explore this possibility, MCF7 cells were treated with etoposide, and endogenous E2F-1, E2F-2 and E2F-3 protein levels were monitored by immunoblotting. E2F-1 protein rapidly accumulates in response to etoposide treatment in contrast to E2F-2 and E2F-3 that are unaffected (Figure 3.2). These results confirm that the induction of E2F-1 protein accumulation is specific for E2F-1. Recent work has shown that the induced accumulation of E2F-1 protein in response to DNA damage does not result from an increase in E2F-1 mRNA (Blattner *et al.*, 1999; Lin *et al.*, 2001), therefore it most likely involves a post-translational mechanism.

### **3.4. Induction of E2F-1 and p53 following DNA damage exhibit similar kinetics**

Accumulation of p53 in response to IR and UV-irradiation is dependent on the ATM and ATR kinases (Kastan and Lim, 2000). More recently Chk2 kinase has also been shown to be involved in the accumulation of p53 in response to IR, as well as regulating its transcriptional activity (Chehab *et al.*, 2000; Takai *et al.*, 2002). To investigate whether E2F-1 exhibited similar kinetics of induction to p53, human MCF7 cells, which express wild-type p53, were treated with DNA damaging agents and the levels of endogenous E2F-1 and p53 were monitored by immunoblotting. Whilst some functional redundancy exists, the radiomimetic drug etoposide, and UV-irradiation are believed to activate different arms of the DNA damage signalling pathway (Durocher and Jackson, 2001). UV-irradiation was able to induce E2F-1 with kinetics closely following the induction of p53 (Figure 3.3b). However in response to etoposide treatment, p53 is induced more rapidly than E2F-1, with p53 levels peaking after 4 hours (Figure 3.3a). As an internal control PCNA was studied, which was not affected by either etoposide or UV treatment and was therefore used as a loading control throughout this study. The observation that both p53 and E2F-1 are induced by the same DNA damaging agents and with similar kinetics, suggests that they may be commonly regulated by the DNA damage signalling pathway.

### 3.5. E2F-1 is regulated by ATM kinase

Induction of E2F-1 in response to DNA damage mediated by ATM-dependent phosphorylation has previously been described (Lin *et al.*, 2001). In order to confirm this observation, the induction of E2F-1 and p53 were examined after treatment with etoposide or UV-irradiation in a cell line deficient in ATM function. Accumulation of p53 in response to IR involves ATM (Siliciano *et al.*, 1997; Chehab *et al.*, 1999; Shieh *et al.*, 1999). Consistent with this, the accumulation of p53 was impaired in response to etoposide in ATM-deficient fibroblasts (Figure 3.4, lanes 2-5, middle). In contrast, p53 was significantly induced by UV-irradiation from as early as 4h after treatment (Figure 3.4, lanes 6-9, middle). Importantly, E2F-1 also failed to be induced by etoposide, in clear contrast to the induction of E2F-1 by UV-irradiation in the same cells (Figure 3.4, compare lanes 2-5 with 6-9, top). As an internal control, PCNA was studied, which was not affected by either etoposide or UV treatments (Figure 3.4, bottom). These results suggest that ATM is required for the proper induction of p53 and E2F-1 in response to DNA d/s breaks, and support the notion that p53 and E2F-1 are commonly regulated by the DNA damage signalling pathway.

### 3.6. E2F-1 is regulated by Chk2 kinase

Given the similarities in the kinetics of induction between E2F-1 and p53, and recent work implicating Chk2, in addition to ATM and ATR kinases, in the regulation of p53 (Chehab *et al.*, 2000; Hirao *et al.*, 2000; Sheih *et al.*,

2000), the role of Chk2 in regulating E2F-1 was investigated. To assess the possibility that E2F-1 is subject to regulation by Chk2 kinase, the effect of a dominant-negative acting derivative of Chk2, (Asp347Ala; Chehab *et al.*, 2000) on the induction of endogenous E2F-1 by DNA damage was studied. Because of the established role of Chk2 kinase in the p53 response (Chehab *et al.*, 2000; Hirao *et al.*, 2000; Sheih *et al.*, 2000), it was anticipated that dominant-negative Chk2 would hinder p53 induction. Western blot analysis confirmed that inhibition of p53 induction was observed in etoposide treated MCF7 cells (Figure 3.5a, compare lanes 3 and 6, middle), with negligible effect on UV treated cells (Figure 3.5a, compare lanes 2 and 5 middle). Most interestingly however, the Chk2 dominant-negative also blocked the induction of E2F-1 in response to etoposide (Figure 3.5a, compare lanes 3 and 6, top), again with negligible effect on UV treated cells (Figure 3.5a, compare lanes 2 and 5, top). The interpretation of these results is affected by the transfection efficiency of Chk2 dominant-negative, nonetheless, these results suggest that Chk2 is involved in signalling DNA damage to E2F-1, specifically in response to agents that induce DNA d/s breaks. In addition these results further strengthen the idea that E2F-1 and p53 are commonly regulated by the DNA damage signalling pathway.

If E2F-1 were to respond to etoposide treatment through the activity of Chk2 kinase, then E2F-1 should fail to be induced by etoposide in cell-types that lack Chk2 activity. To address this idea the regulation of E2F-1 in HCT-15 colon carcinoma cells, which express mutant p53 and Chk2 mutated in the FHA domain (Phe145Trp) which prevents Chk2 from engaging in phosphorylation dependent protein-protein interactions was explored (Bell *et al.*, 1999; Falck *et*

*et al.*, 2001b). Western blot analysis showed that E2F-1 was not induced by etoposide (Figure 3.5b, lanes 4-6, top), in contrast to the induction of E2F-1 by UV-irradiation in the same cells (Figures 3.5b, lanes 1-3, top). The mutation in p53 prevents its induction in these cells (Figure 3.5b, lanes 1-6, middle), and PCNA remains unaltered by either etoposide or UV treatments (Figure 3.5b, bottom). Taken together with the previous results on the effects of dominant-negative Chk2, which in MCF7 cells blocked the induction of E2F-1 by etoposide but not by UV-irradiation (Figure 3.5a), these results suggest a physiological role for Chk2 in regulating the response of E2F-1 to etoposide.

Further evidence for this idea was gained by introducing wild-type Flag-tagged Chk2 into either U2OS (carrying low levels of endogenous Chk2) or HCT-15 cells and measuring the level of endogenous E2F-1 after etoposide treatment by western blotting. In U2OS cells the introduction of Chk2 allowed a greater level of E2F-1 to be induced by etoposide (Figure 3.5c, compare lanes 2 and 4, top). In HCT-15 cells, introducing wild-type Chk2 re-instated the ability of E2F-1 to respond to etoposide (Figure 3.5d, compare lanes 1-3 with 4-6, top). Under these conditions, recognition of exogenous Chk2 by an antibody specific for phosphorylated Thr68 residue, which is required for Chk2 to become catalytically active (Matsuoka *et al.*, 2000; Melchionna *et al.*, 2000; Ahn *et al.*, 2000), occurred in etoposide treated cells, and correlated with the induction of E2F-1 (Figure 3.5d, lanes 5 and 6, upper bottom). Moreover, a slower mobility form of Chk2 was recognised by a Flag specific antibody in etoposide treated cells (Figure 3.5d, lanes 5 and 6, middle). These results support a role for Chk2 in regulating E2F-1 in response to etoposide, and concur with the earlier result derived from dominant-negative Chk2 (Figure 3.5a).

### 3.7. Conclusions

Data presented in this chapter demonstrates that E2F-1 and p53 are commonly regulated by the DNA damage signalling pathway. Consistent with reports by others (Blattner *et al.*, 1999; O'connor *et al.*, 2000; Lin *et al.*, 2001), an induction of E2F-1 by several DNA damaging chemotherapeutic drugs in a variety of cell lines irrespective of p53 or pRb status was observed. Induction is rapid and specific to E2F-1, and is mediated through a post-transcriptional mechanism that likely involves protein stabilisation (Blattner *et al.*, 1999; Lin *et al.*, 2001).

The role of ATM in the induction of E2F-1 protein in response to DNA damage, in particular agents that cause d/s breaks, has been confirmed, and several lines of evidence are presented that establish Chk2 in the control of E2F-1 protein accumulation following DNA damage. First, a Chk2 dominant-negative mutant blocked E2F-1 induction in response to etoposide treatment, but not UV-irradiation. In addition, E2F-1 failed to accumulate in response to etoposide in HCT-15 cells that lack Chk2 activity. Further evidence was gained by introducing wild-type Chk2 into either U2OS cells (low levels of Chk2), or HCT-15 cells lacking Chk2 activity. In U2OS cells the introduction of Chk2 allowed a greater level of E2F-1 to be induced by etoposide, while in HCT-15 cells, introduction of wild-type Chk2 restored the ability of E2F-1 to respond to etoposide. Therefore, based on these results, the induction of E2F-1 in response to DNA damage, specifically agents that induce DNA d/s breaks, involves signalling through ATM and Chk2 kinases.

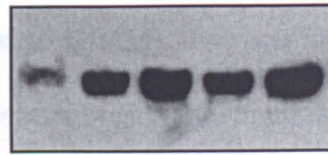
### **Figure 3.1. Induction of E2F-1 in tumour cells following DNA damage**

Western blot analysis of E2F-1 from various human cell lines after 16 h treatment with 50 J/m<sup>2</sup> UV-irradiation (UV), 10μM etoposide (Et), 10μM cisplatin (Cis), and 10μM doxorubicin (Dox), shows induction of E2F-1 regardless of their p53 or pRb status. Extracts from U2OS cells (p53 wild-type, pRb hyper-phosphorylated), SAOS2 cells (*p53*<sup>-/-</sup> with a C-term truncated pRb), MCF7 cells (p53 wild-type, pRb hyper-phosphorylated) or T98G cells (p53 wild-type, pRb wild-type) were resolved by denaturing gel electrophoresis and immunoblotted for endogenous E2F-1 using KH-95 antibody. Equal amounts of total protein were loaded in each lane as determined by Ponceau S staining.

Figure 3.2. Specific induction of E2F1 following DNA damage

MCF7

DNA damage : - UV Et Cis Dox



E2F-1

1 2 3 4 5

T98G

DNA damage : - UV Et Cis Dox

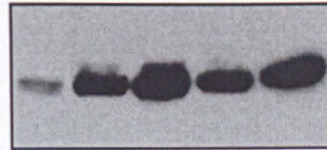


E2F-1

1 2 3 4 5

SAOS2

DNA damage : - UV Et Cis Dox

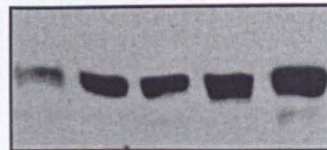


E2F-1

1 2 3 4 5

U2OS

DNA damage : - UV Et Cis Dox



E2F-1

1 2 3 4 5



### **Figure 3.2. Specific induction of E2F-1 following DNA damage**

Western blot analysis of E2F-1, E2F-2 and E2F-3 protein accumulation in human cells exposed to etoposide (10 $\mu$ M). Extracts from MCF7 cells were resolved by denaturing gel electrophoresis and immunoblotted for endogenous E2F-1 (top), E2F-2 (middle), and E2F-3 (bottom). Equal amounts of total protein were loaded in each lane as determined by Ponceau S staining.

Figure 3.1. E2F-1 accumulation following UV-B exposure and retA

Fig 3.1

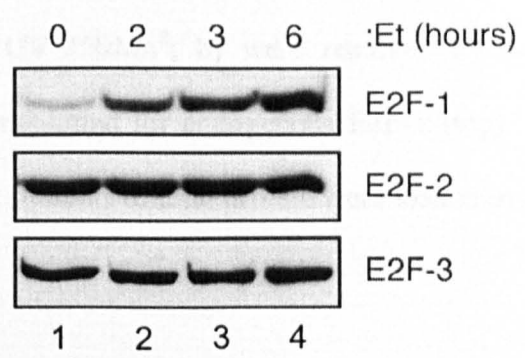
Western blot analysis of E2F-1 and E2F-2 and E2F-3

expressed in response to UV-B irradiation. Extracts from MCF-7 cells were

exposed to retA (10<sup>-6</sup>M) for

electrophoresis and

in PCNA (cellular



**Figure 3.3. E2F-1 accumulates following DNA damage with similar kinetics to p53**

a) and b) Western blot analysis of E2F-1 and p53 protein accumulation in cells exposed to etoposide or UV-irradiation. Extracts from MCF7 cells exposed to etoposide (10 $\mu$ M; a) or UV (50J/m<sup>2</sup>; b) were resolved by denaturing gel electrophoresis and immunoblotted for endogenous E2F-1 (top), p53 (middle) or PCNA (bottom). Equal amounts of total protein were loaded in each lane.

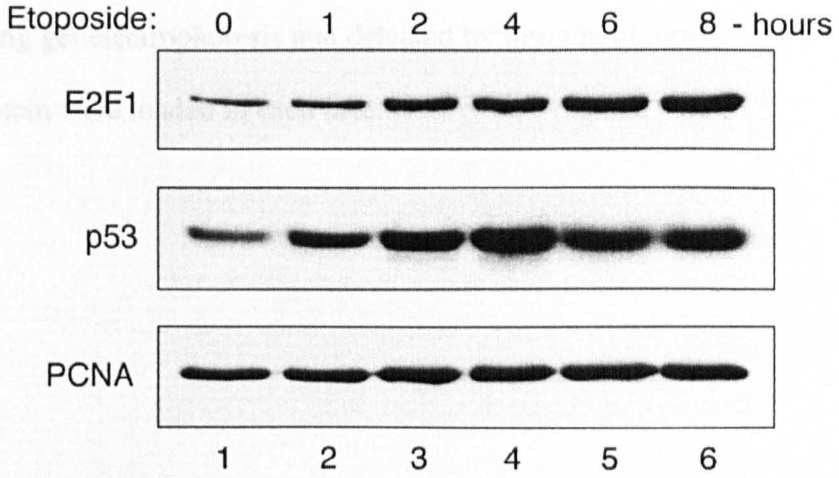
Figure 3.4. E2F-1 accumulation = A. Cell fractionation in response to etoposide

AT-Grobbins (G942350) were exposed to ultraviolet (254 nm) for 10 min

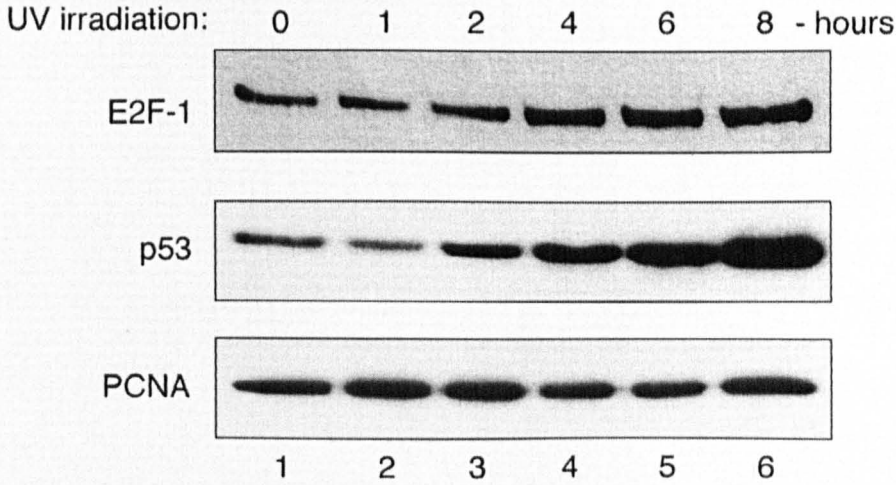
irradiation (5000 J) and cell extracts were prepared 1, 2, 3, 4, 6, and 8 hours

after etoposide (100 nM) treatment. E2F-1 (top), p53 (middle), and PCNA (bottom) were detected by Western blotting

a)



b)

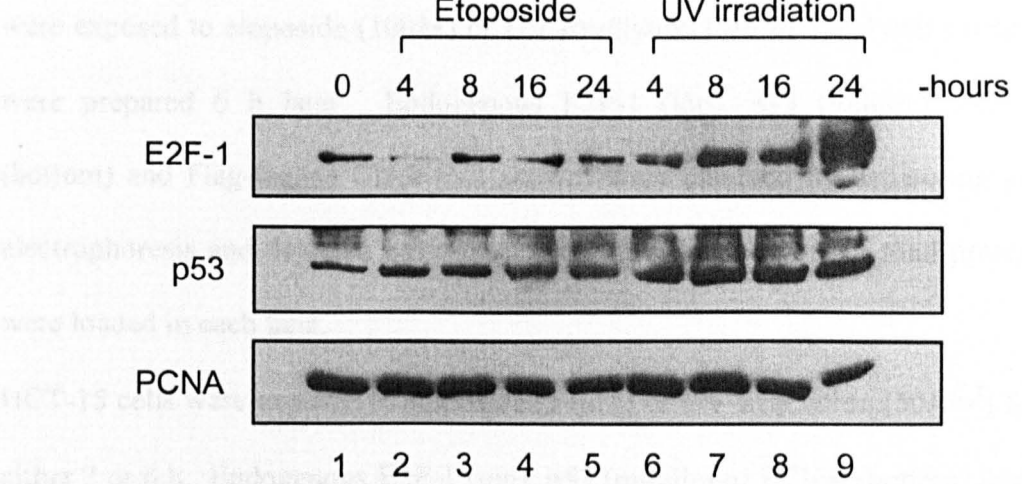


### **Figure 3.4. E2F-1 accumulation is ATM dependent in response to etoposide**

AT-fibroblasts (GM02350) were exposed to etoposide (10 $\mu$ M) or UV-irradiation (50J/m<sup>2</sup>) and cell extracts were prepared 4, 8, 16 or 24 h later. Endogenous E2F-1 (top), p53 (middle), and PCNA (bottom) were resolved by denaturing gel electrophoresis and detected by immunoblotting. Equal levels of total protein were loaded in each lane.

Western blot analysis of E2F-1 and p53 levels in HCT-15 cells treated with Etoposide or UV irradiation.

The cells were treated with Etoposide or UV irradiation for the indicated times. The cell lysates were extracted and analyzed by Western blotting.



Western blot analysis of E2F-1 and p53 levels in HCT-15 cells treated with Etoposide or UV irradiation.

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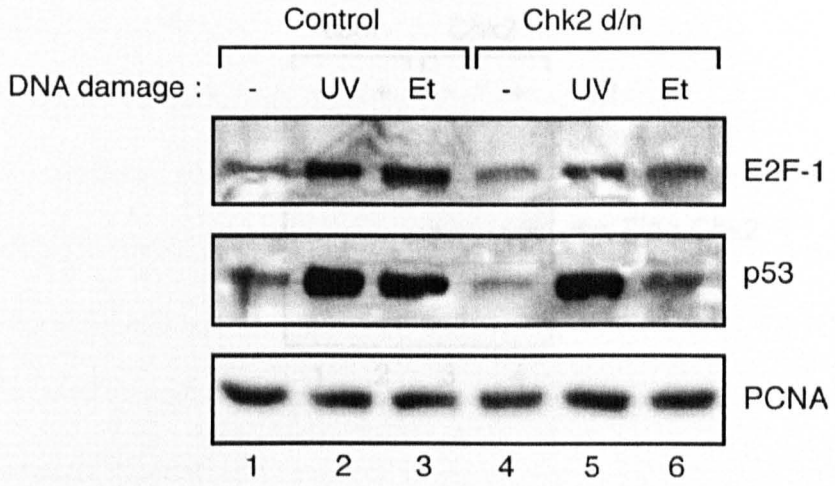
The cells were treated with Etoposide or UV irradiation for the indicated times. The cell lysates were extracted and analyzed by Western blotting.

Western blot analysis of E2F-1 and p53 levels in HCT-15 cells treated with Etoposide or UV irradiation.

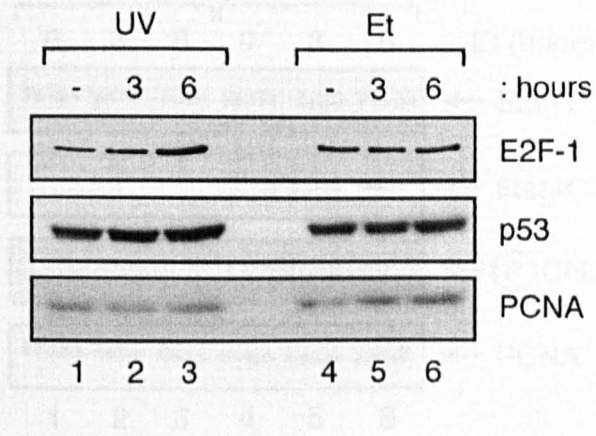
**Figure 3.5. E2F-1 accumulates following DNA damage and requires Chk2**

- a) MCF7 cells were transfected with plasmids expressing Flag-tagged Chk2 dominant-negative protein (5 $\mu$ g), or vector alone (5 $\mu$ g) as indicated. The cells were exposed to etoposide (10 $\mu$ M) or UV-irradiation (50J/m<sup>2</sup>) and cell extracts were prepared 6 h later. Endogenous E2F-1 (top), p53 (middle), PCNA (bottom) and Flag-tagged Chk2 (not shown) were resolved by denaturing gel electrophoresis and detected by immunoblotting. Equal levels of total protein were loaded in each lane.
- b) HCT-15 cells were exposed to etoposide (10 $\mu$ M) or UV-irradiation (50J/m<sup>2</sup>) for either 3 or 6 h. Endogenous E2F-1 (top), p53 (middle) or PCNA (bottom) were resolved by denaturing gel electrophoresis and detected by immunoblotting. Equal levels of total protein were loaded in each lane.
- c) U2OS cells were transfected with 5 $\mu$ g vector alone (tracks 1 and 2) or flag-tagged Chk2 (tracks 3 and 4) and exposed to 10 $\mu$ M etoposide for 6 h. Extracts were prepared and immunoblotted for endogenous E2F-1 (top), Flag-tagged Chk2 (middle) and PCNA (bottom) as described.
- d) HCT-15 cells were transfected with 5 $\mu$ g vector alone (tracks 1, 2 and 3) or Flag-tagged Chk2 (tracks 4, 5 and 6) and exposed to 10 $\mu$ M etoposide for 3 or 6 h. Extracts were prepared and immunoblotted for endogenous E2F-1 (top), Flag-tagged Chk2 (lower), phosphorylated Thr68 Chk2 (upper bottom), and PCNA (bottom) as described.

a)



b)

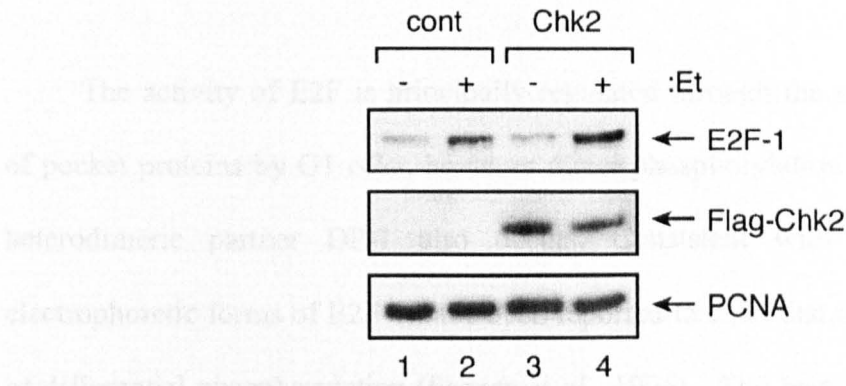




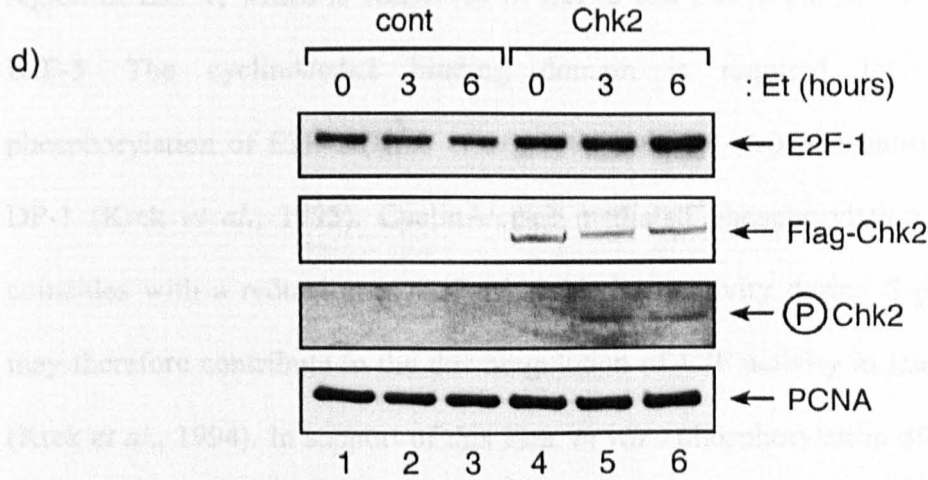
# Chapter 4. Phosphorylation of E2F-1

## 4.1. introduction

c)



d)



# Chapter 4. Phosphorylation of E2F-1

## 4.1. Introduction

The activity of E2F is principally regulated through the phosphorylation of pocket proteins by G1 cdks, however direct phosphorylation of E2F and its heterodimeric partner DP-1 also occurs. Consistent with this, multiple electrophoretic forms of E2F-1 have been reported to exist that are the products of differential phosphorylation (Peeper *et al.*, 1995). The best characterised is the phosphorylation of E2F-1 and DP-1 by cyclinA/cdk2 kinase. E2F-1 and DP-1 are efficiently phosphorylated *in vitro* by cyclinA/cdk2 (Dymlacht *et al.*, 1994; Kitagawa *et al.*, 1995), and cyclinA/cdk2 binds to a domain in the N-terminal region of E2F-1, which is conserved in E2F-2 and E2F-3 but not in E2F-4 or E2F-5. The cyclinA/cdk2 binding domain is required for *in vitro* phosphorylation of E2F-1 (Krek *et al.*, 1994), and *in vivo* phosphorylation of DP-1 (Krek *et al.*, 1995). CyclinA/cdk2 mediated phosphorylation of DP-1 coincides with a reduction in E2F DNA-binding activity during S-phase and may therefore contribute to the downregulation of E2F activity in late S-phase (Krek *et al.*, 1994). In support of this idea, *in vitro* phosphorylation of the E2F-1/DP-1 heterodimer by cyclinA/cdk2 reduced its ability to bind DNA (Dymlacht *et al.*, 1994; Xu *et al.*, 1994; Kitagawa *et al.*, 1995), and overexpression of E2F-1 with a phosphorylation deficient DP-1 caused a prolonged S-phase followed by apoptosis (Krek *et al.*, 1995).

In addition, phosphorylation of E2F-1 on Ser375, one of the sites phosphorylated by cyclinA/cdk2 greatly enhanced its affinity for pRb *in vitro* (Peeper *et al.*, 1995), however no *in vivo* significance of phosphorylation at this site has been determined. Conversely, phosphorylation of Ser332 and Ser337 in the C-terminal half of E2F-1 prevent E2F-1 from binding to pRb *in vivo* (Fagan *et al.*, 1994), although the kinase responsible has not yet been identified.

It has also been demonstrated that phosphorylation of E2F-1 on Ser403 and Thr433 can be mediated by a kinase associated with TFIIH, and results in the down-regulation of E2F-1 stability (Vandel and Kouzarides., 1999), and E2F-5 can be phosphorylated on Thr251 by cyclinE/cdk2 which augments the recruitment of the transcriptional co-activator p300 stimulating activation of E2F responsive genes in late G1 phase (Morris *et al.*, 2000).

More recently, E2F-1 was shown to be phosphorylated on Ser31 in an ATM-dependent manner in response to DNA damage (Lin *et al.*, 2001). Ser31 was shown to be important for the stabilisation of E2F-1, and induction of apoptosis in mouse thymocytes (Lin *et al.*, 2001).

Data presented in the previous chapter showed that E2F-1 stabilisation is regulated by both ATM and Chk2 kinases in response to DNA damage. In this chapter the phosphorylation of E2F-1 by Chk2 and the identification of potential phosphorylated residue(s) are explored.

## **Results**

### **4.2. Site-directed mutagenesis of E2F-1**

To further explore the mechanism for the involvement of Chk2 in E2F-1 stabilisation, the possibility that E2F-1 might be a direct substrate for Chk2 kinase activity was investigated. Chk kinases are Ser/Thr-specific kinases for which a small number of physiological substrates have been identified (Bartek and Lukas, 2003). A consensus Chk phosphorylation site found in other substrates has been determined (Hutchins *et al.*, 2000; O'Neill *et al.*, 2002; Seo *et al.*, 2003). The consensus amino-acid sequence for phosphorylation by Chk kinases is  $\phi$ -X- $\beta$ -X-X-(S/T), where  $\phi$  is a hydrophobic residue,  $\beta$  is a basic residue and X is any amino-acid. This motif was used to search the E2F-1 sequence and found, in the C-terminal region of the protein centred on Ser364, a site that closely resembled the consensus Chk phosphorylation site (Fig. 4.1a and 4.1b). The serine at 364 is unique to E2F-1, and the sequence surrounding Ser364 was more similar to the Cdc25, PML and BRCA1 sites than to the p53 site (Fig. 4.1a).

To investigate whether E2F-1 is regulated by Chk2 phosphorylation, a mutational analysis was performed at the potential Chk2 phosphorylation site. A mutant E2F-1 derivative in which Ser364 was altered to an alanine (A) residue (from now on referred to as S364A) was prepared. Alanine substitution results in the replacement of the OH group in serine with a CH<sub>3</sub> side chain, destroying the potential Chk2 phosphorylation site (Fig.4.1b). Furthermore, being a small, uncharged residue it is less likely than other amino acids to cause conformational changes to a protein. After sequencing confirmed that Ser364

had been successfully substituted with alanine, over-expression in U2OS cells and western blot analysis of cell extracts confirmed that the S364A mutant was expressed to a similar level as E2F-1 (Fig. 4.1c, compare lanes 2 and 3).

### **4.3. E2F-1 is phosphorylated *in vitro* by Chk2**

To explore the idea that E2F-1 is phosphorylated by Chk2, *in vitro* phosphorylation reactions were performed with recombinant E2F-1 proteins. E2F-1 and the mutant derivative, S364A, were expressed as GST-fusion proteins in bacteria. When the purified preparations were subjected to SDS-PAGE and coomassie blue staining, a 96kDa band, the predicted size of the GST-fusion protein, was detected (Figure 4.2a). In addition a smaller band was detected, probably as a result of protein degradation or bacterial contamination in the preparation. The identity of full-length E2F-1 was confirmed by immunoblotting with an anti-E2F-1 antibody. A strong band at 96 kDa, was detected (Figure 4.2b).

*In vitro* kinase assays were performed using GST-E2F-1 and either His-tagged Chk2 purified from bacteria, or cyclinA/cdk2 purified from baculovirus infected *Sf9* cells. Initially, *in vitro* kinase assays supplemented with unlabelled, cold ATP were performed. Immunoblotting the reaction products with an anti-E2F-1 antibody revealed that a slower mobility form of E2F-1 was evident only with the wild-type protein upon addition of Chk2 kinase and a supply of ATP, and was abrogated with the S364A mutant (Fig. 4.2c, compare lanes 2 and 4). As a control the specificity of the S364A mutant was assessed by determining whether it could be phosphorylated by another kinase, namely cyclinA/cdk2,

which phosphorylates E2F-1 (Krek *et al.*, 1994). E2F-1 and S364A were phosphorylated at about equivalent efficiency by cyclinA/cdk2 (Fig. 4.2d, compare lanes 2 and 4), as estimated from the amount of slower mobility E2F protein, suggesting that the absence of S364A phosphorylation results from altering a specific Chk2 kinase site.

Next *in vitro* kinase assays supplemented with  $\gamma$ -<sup>32</sup>P were performed. Again Chk2 was able to phosphorylate E2F-1 when reactions were supplemented with a source of ATP (Figure 4.2e, compare lanes 1 and 2). The efficiency of phosphorylation was similar to the level observed for an established Chk2 substrate, namely Cdc25C (Fig. 4.2e, compare lanes 2 and 5). Importantly, the S364A mutant derivative failed to be phosphorylated (Fig. 4.2e, lane 3), suggesting that Ser364 is phosphorylated by Chk2 kinase. As a control, the specificity of the S364A mutant was again assessed by determining whether it could be phosphorylated by cyclinA/cdk2. E2F-1 and S364A were phosphorylated at about equivalent efficiency by cyclinA/cdk2 (Fig. 4.2f, compare lanes 2 and 3), confirming that the absence of S364A phosphorylation results from altering a specific Chk2 kinase site.

#### **4.4. Generation of a phospho-Ser364 specific antibody**

To confirm that Ser364 is phosphorylated by Chk2 kinase, an anti-phospho-specific peptide antiserum directed against the consensus site in which phosphorylated Ser364 resides was prepared (referred to as P-S364; Fig. 4.3a).

First of all the reactivity of the crude P-S364 was assessed on *in vitro* phosphorylated E2F-1. P-S364 reacted strongly with Chk2 phosphorylated E2F-

1 compared to unphosphorylated E2F-1 (Figure 4.3b, compare lanes 3 and 4), in contrast to a general E2F-1 antibody which exhibited similar reactivity to both un- and phosphorylated E2F-1 (Fig. 4.3b, compare lanes 1 and 2). Additionally, P-S364 had greatly reduced activity on the S364A mutant derivative (Fig. 4.3c, compare lanes 2 and 4, top). Importantly, the reactivity of the antiserum for phosphorylated Ser364 E2F-1 was specific, as the signal was competed only by inclusion of the phosphorylated E2F-1 peptide, and not by the unphosphorylated E2F-1 peptide (Figure 4.3d, compare lanes 1 and 2, top). These results support the view that the Ser364 site is phosphorylated by Chk2 kinase, and confirm that P-S364 recognises the phosphorylated site in E2F-1.

#### **4.5. E2F-1 is phosphorylated on Ser364 *in vivo***

Whilst E2F-1 was found to be phosphorylated by Chk2 *in vitro*, it was important to substantiate these findings *in vivo*. After peptide affinity purification (see Materials and Methods), the reactivity of the P-S364 antiserum was very much more specific for the phosphorylated Ser364 peptide compared to the non-phosphorylated Ser364 peptide (Fig. 4.4a). Furthermore, purified P-S364 reacted strongly *in vitro* with Chk2 phosphorylated E2F-1 compared to unphosphorylated E2F-1 (Figure 4.4b, compare lanes 1 and 3, top). Importantly the affinity purified P-S364 showed no reactivity on the S364A mutant (Figure 4.4b, lane 2, top). In contrast, a general E2F-1 antibody exhibited similar reactivity to both un- and phosphorylated E2F-1 (Figure 4.4b, bottom).

Purified P-S364 was then used to study the regulation of endogenous E2F-1 under DNA damage conditions, to ascertain whether Ser364 is subject to

phosphorylation control in cells. MCF7 cells were treated with etoposide for 2 h and E2F-1 was monitored by western blot analysis with P-S364. The reactivity of P-S364 was compared to the general anti-E2F-1 antibody (KH-95). Under these experimental conditions, there was little change in the steady-state level of E2F-1 when measured with the general E2F-1 antibody (Fig.4.4c, compare lanes 1 and 3). In non-stressed MCF7 cells P-S364 exhibited no activity, in contrast to etoposide-treated MCF7 cells where the antiserum clearly reacted with E2F-1 (Fig. 4.4c, compare lanes 2 and 4). Furthermore, when antibody that had been affinity purified using the phosphorylated Ser364 peptide was used on the same cell extracts, E2F-1 was detected only in etoposide treated cells (Fig. 4.4d, compare lanes 1 and 2, top). In contrast, the phosphorylated Ser364 peptide affinity depleted antiserum ( $\alpha$ S364), which should include antibodies against the non-phosphorylated Ser364 residue, recognised an equivalent level of E2F-1 in treated and untreated cells (Fig. 4.4d, compare lanes 3 and 4, top). To ensure that the polypeptide recognised by P-S364 in extracts of etoposide treated cells was E2F-1, immunoprecipitation assays were performed with the general E2F-1 antibody, KH-95, prior to western blot analysis. Immunoprecipitated E2F-1 from etoposide treated cells was clearly recognised by P-S364, in contrast to E2F-1 from non-stressed cells (Fig. 4.4e, compare lanes 3 and 4, top). These results support the idea that Ser364 on E2F-1 undergoes DNA damage-responsive phosphorylation *in vivo*.



## 4.6. Conclusions

Chk2 is an Ser/Thr-specific kinase for which a small number of physiological substrates have been identified (Bartek and Lukas, 2003). A consensus sequence motif for phosphorylation, derived from Chk phosphorylation sites in different substrates has recently been identified (Hutchins *et al.*, 2000; O'Neill *et al.*, 2002; Seo *et al.*, 2003). In this chapter, the consensus sequence motif was used to screen the amino-acid sequence of E2F-1 and, in the C-terminal region of E2F-1 centred on serine residue 364, a site was identified that fitted the consensus phosphorylation site.

The importance of the hypothetical Chk2 phosphorylation site was explored by site-directed mutagenesis. E2F-1 served as an *in vitro* substrate for Chk2 kinase, whereas a mutant E2F-1 derivative in which Ser364 was altered to an alanine residue failed to be phosphorylated, suggesting that Ser364 is the only target site on E2F-1 phosphorylated by Chk2 kinase.

Further evidence that Chk2 phosphorylates Ser364 was gained by preparing an anti-phospho-specific peptide antibody directed against the phosphorylated Ser364 residue. *In vitro* the P-S364 antibody reacted strongly with Chk2 phosphorylated E2F-1 compared to unphosphorylated E2F-1, and showed little or no activity towards the S364A mutant. E2F-1 is also phosphorylated on the same site *in vivo* in response to DNA damage, illustrated by the observation that in etoposide treated MCF7 cells, the P-S364 antiserum clearly reacted with E2F-1, in contrast to E2F-1 from non-stressed cells. Thus, Ser364 on E2F-1 undergoes DNA damage-responsive phosphorylation in cells in a Chk2 dependent manner.

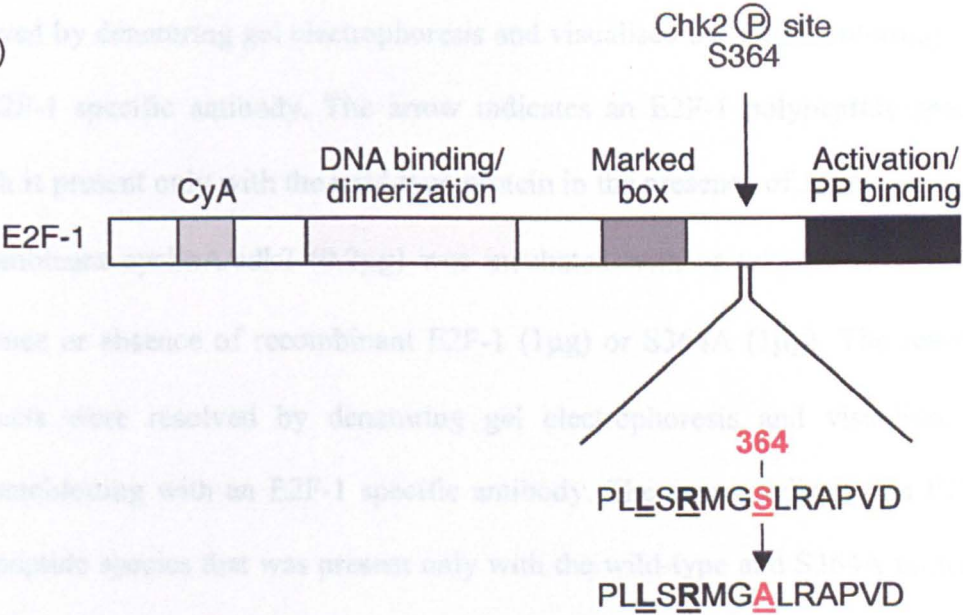
#### **Figure 4.1. Identification of a Chk2 kinase phosphorylation site in E2F-1**

- a) Comparison of the Chk2 phosphorylation sites in Cdc25A, Cdc25C, BRCA1, PML and p53 with E2F-1. Residues in red form the consensus Chk2 kinase site. Residues that fall within the consensus Chk2 kinase site are in bold.
- b) Diagram of E2F-1 together with relevant protein domains. The location of the Chk2 phosphorylation site is indicated between the marked box and activation/pocket protein (PP) binding domain. The serine residue (S364) that was mutated to alanine (A) is shown, the mutant derivative being referred to as S364A. CyA (cyclinA-binding domain).
- c) U2OS cells were co-transfected with expression vectors for CMV-DP-1 (1µg) and either HA-tagged E2F-1 (5µg) or HA-tagged S364A (5µg). CMV-β-gal was also transfected to monitor transfection efficiency. Extracts were prepared from transfected cells and subjected to immunoblotting with a HA-specific monoclonal antibody.

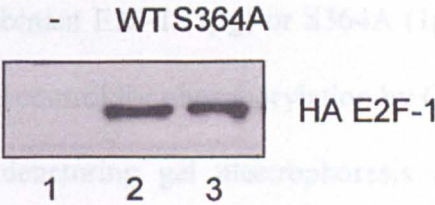
a)

Cdc25A : 116 P A L K R S H S D S L D H D 129  
Cdc25C : 209 S G L Y R S P S M P E N L N 222  
PML : 110 E S L Q R R L S V C R P I V 123  
E2F-1 : 357 P L L S R M G S L R A P V D 371  
BRCA1 : 981 P P L F P I K S F V K T K C 994  
p53 : 13 P L S Q E T F S D L W K L L 26

b)



c)



#### **Figure 4.2. Chk2 phosphorylates E2F-1 *in vitro***

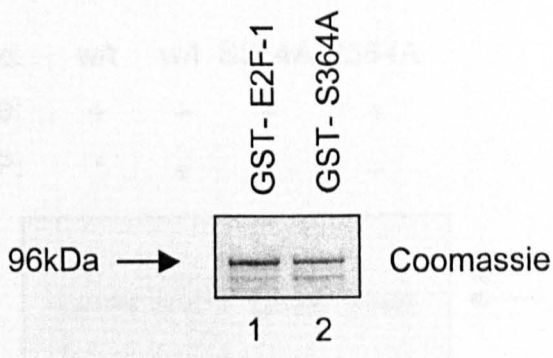
- a) Recombinant E2F-1 and S364A (1µg) were resolved by denaturing gel electrophoresis and stained with coomassie blue.
- b) Recombinant E2F-1 and S364A (100ng) were resolved by denaturing gel electrophoresis and analysed by western blotting with an E2F-1 specific antibody (KH-95).
- c) Recombinant Chk2 (0.2µg) was incubated with or without ATP in the presence or absence of recombinant E2F-1 (1µg) or S364A (1µg). The reaction products were resolved by denaturing gel electrophoresis and visualised by immunoblotting with an E2F-1 specific antibody. The arrow indicates an E2F-1 polypeptide species which is present only with the wild-type protein in the presence of ATP.
- d) Recombinant cyclinA/cdk2 (0.2µg) was incubated with or without ATP in the presence or absence of recombinant E2F-1 (1µg) or S364A (1µg). The reaction products were resolved by denaturing gel electrophoresis and visualised by immunoblotting with an E2F-1 specific antibody. The arrow indicates an E2F-1 polypeptide species that was present only with the wild-type and S364A proteins, but only in the presence of ATP.
- e) Recombinant Chk2 (0.2µg) was incubated with or without <sup>32</sup>P ATP in the presence or absence of recombinant E2F-1 (1µg) or S364A (1µg). Recombinant Cdc25C was used as a positive control for phosphorylation by Chk2. The reaction products were resolved by denaturing gel electrophoresis and visualised by autoradiography. Phosphorylated E2F-1, auto-phosphorylated Chk2 and phosphorylated Cdc25C are indicated.

- f) Recombinant cyclinA/cdk2 (0.2µg) was incubated with or without  $^{32}\text{P}$  ATP in the presence or absence of recombinant E2F-1 (1µg) or S364A (1µg). Recombinant pRb was used as positive control for cyclinA/cdk2 activity (not shown). The reaction products were resolved by denaturing gel electrophoresis and visualised by autoradiography. Phosphorylated E2F-1 and auto-phosphorylated Cdk2 are indicated.

a)

Phosphorylation by Cdk 2

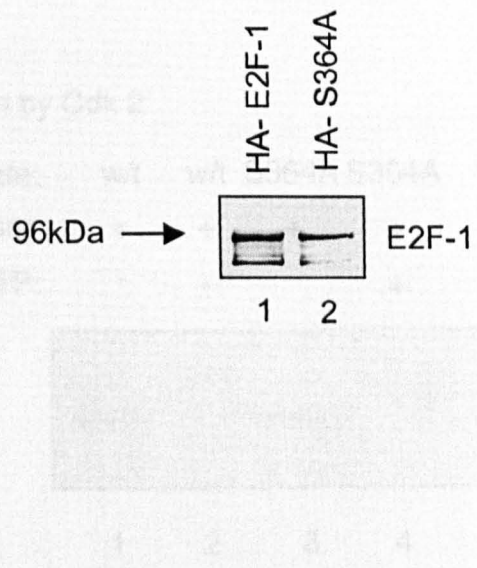
substrate	wt	wt S364A
Cdk2 kinase	+	+
ATP	+	+



b)

Phosphorylation by Cdk 2

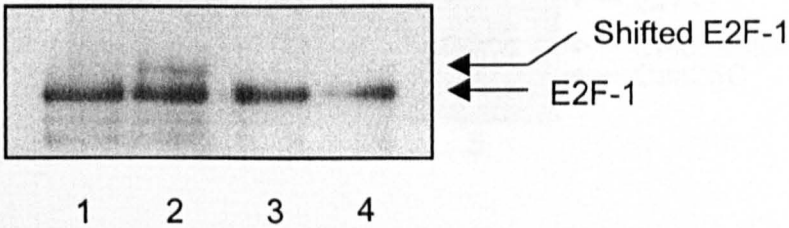
substrate	wt	wt S364A
Cdk2 kinase	+	+
ATP	+	+



c)

Phosphorylation by Chk 2

substrate:	w/t	w/t	S364A	S364A
Chk2 kinase:	+	+	+	+
ATP:	-	+	-	+



d)

Phosphorylation by Cdk 2

substrate:	w/t	w/t	S364A	S364A
Cdk2 kinase:	+	+	+	+
ATP:	-	+	-	+

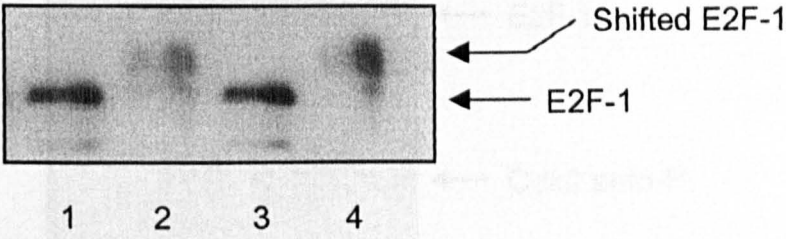


Figure 4.3. E2F-1 is phosphorylated by Chk2 in vitro.

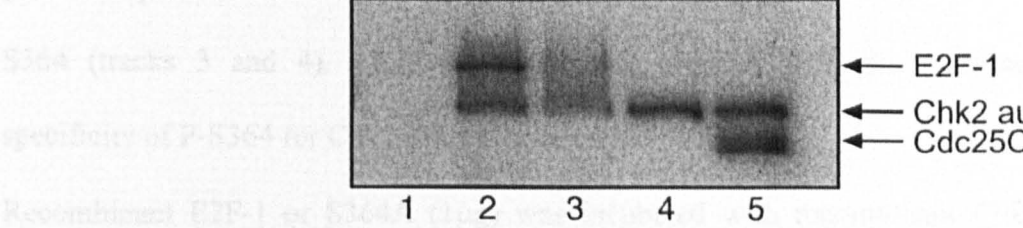
a) Sequence of phosphopeptide derived from E2F-1 used to generate Phos-1 specific antiserum. Bold letters indicate the residues used for the antiserum.

b) Recombinant Chk2 (0.2 µg) was incubated with recombinant E2F-1 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

c) Recombinant E2F-1 or S364A (0.2 µg) was incubated with recombinant Chk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

d) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Chk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

e) Phosphorylation by Chk 2



f) Phosphorylation by Cdk 2

g) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

h) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

i) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

j) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

k) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

l) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

m) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

n) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

o) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

p) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

q) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

r) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

s) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

t) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.

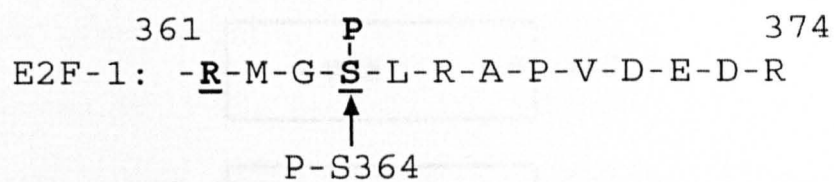
u) Recombinant E2F-1 (0.2 µg) was incubated with recombinant Cdk2 (0.2 µg) in the presence of ATP (50 mM) for 10 min. The reaction was stopped by adding Laemmli buffer and the products were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G250.



**Figure 4.3. E2F-1 is phosphorylated on Ser364 *in vitro***

- a) Sequence of phospho-peptide derived from E2F-1 used to prepare the phospho-specific antiserum. Bold letters indicate the residues that fall into the consensus Chk phosphorylation site.
- b) Recombinant Chk2 (0.2µg) was incubated with recombinant E2F-1 (1µg) in the presence or absence of ATP (50µM). The reaction products were resolved by gel electrophoresis and immunoblotted with either KH95 (tracks 1 and 2) or P-S364 (tracks 3 and 4). E2F-1 is indicated, together with the increased specificity of P-S364 for Chk2 phosphorylated E2F-1.
- c) Recombinant E2F-1 or S364A (1µg) was incubated with recombinant Chk2 (0.2µg) in the presence or absence of ATP (50µM). The upper blot was treated with affinity purified P-S364 antibodies, and the lower blot with the general E2F-1 antibody KH-95.
- d) Recombinant E2F-1 (1µg) was incubated with recombinant Chk2 (0.2µg) supplemented with ATP (50µM), in the presence of either the phosphorylated or non-phosphorylated Ser364 peptide (1µg). The reaction products were resolved by denaturing gel electrophoresis and immunoblotted with P-S364 antibodies (top), or the general E2F-1 antibody KH-95 (bottom).

a)



b)

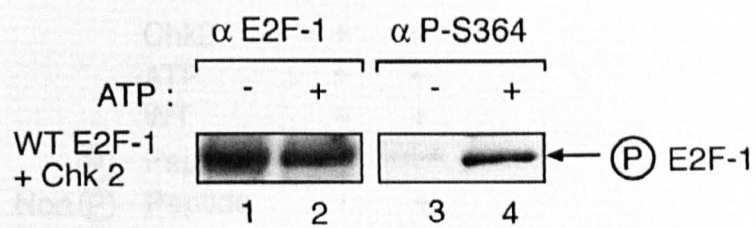
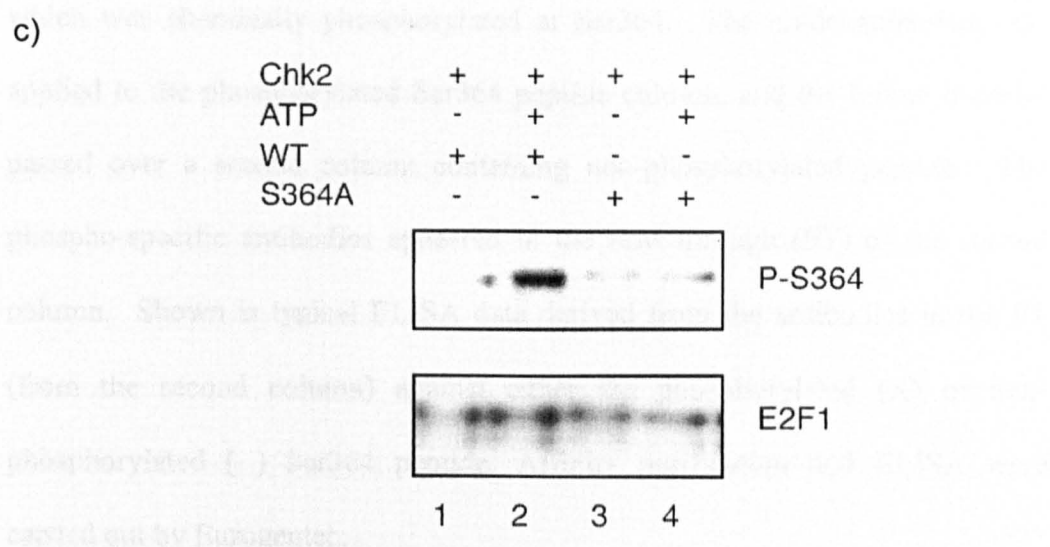


Figure 4.4: E2F1 is phosphorylated on Ser364 in cells

Two preparations were generated, each on the same day, and each with a different

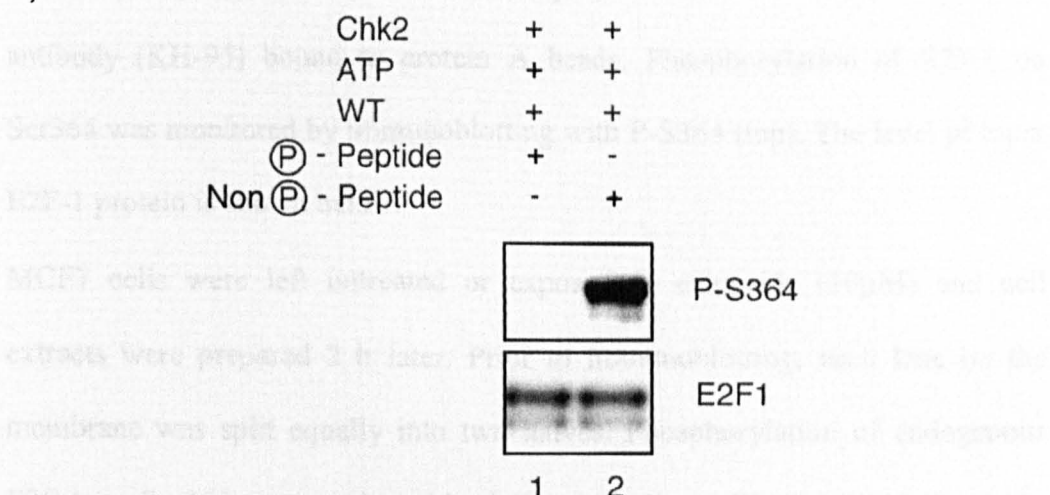
condition. The first preparation was generated at 100% confluence, the second



b) Recombinant Chk2 (0.2 µg) was incubated with recombinant E2F1 (1 µg) and

S364A (1 µg), in the presence or absence of ATP (0.2 µg) for 10 min.

d) Immunoprecipitation of E2F1; reaction products were analyzed with P-S364



E2F1 on Ser364 was monitored by immunoblotting with the anti-phospho-S364

antibody P-S364 (tracks 2 and 4). The amount of extract used was similar

levels of total E2F1 as shown by immunoblotting with antibody E2F1 (tracks

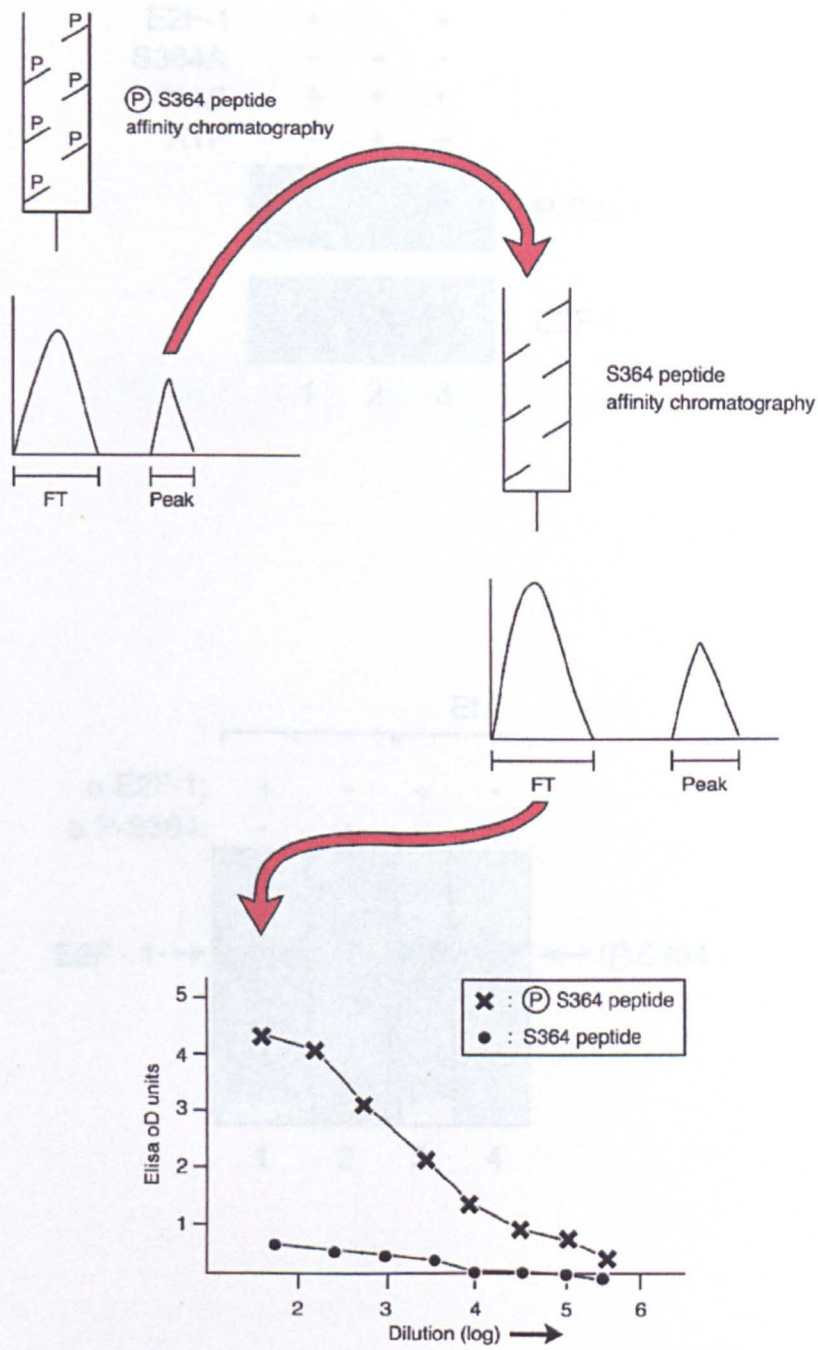
1 and 3).

**Figure 4.4. E2F-1 is phosphorylated on Ser364 *in vivo***

- a) Two peptides were generated based on the sequence around Ser364, one of which was chemically phosphorylated at Ser364. The crude antiserum was applied to the phosphorylated Ser364 peptide column, and the bound material passed over a second column containing non-phosphorylated peptide. The phospho-specific antibodies appeared in the flow-through (FT) of the second column. Shown is typical ELISA data derived from the antibodies in the FT (from the second column) against either the phosphorylated (X) or non-phosphorylated ( ) Ser364 peptide. Affinity purification and ELISA were carried out by Eurogentec.
- b) Recombinant Chk2 (0.2 $\mu$ g) was incubated with recombinant E2F-1 (1 $\mu$ g) or S364A (1 $\mu$ g), in the presence or absence of ATP (50 $\mu$ M). For immunoprecipitation of E2F-1, reaction products were incubated with E2F-1 antibody (KH-95) bound to protein A beads. Phosphorylation of E2F-1 on Ser364 was monitored by immunoblotting with P-S364 (top). The level of input E2F-1 protein is shown below.
- c) MCF7 cells were left untreated or exposed to etoposide (10 $\mu$ M) and cell extracts were prepared 2 h later. Prior to immunoblotting, each lane on the membrane was split equally into two halves. Phosphorylation of endogenous E2F-1 on Ser364 was monitored by immunoblotting with the phospho-specific antibody P-S364 (tracks 2 and 4). The amounts of extracts used have similar levels of total E2F-1 as shown by immunoblotting with antibody KH95 (tracks 1 and 3).

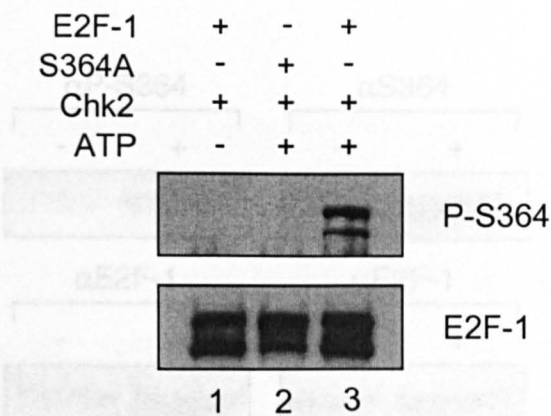
- d) MCF7 cells were treated as described in a), and probed with either affinity purified P-S364 (top left) or the affinity-depleted antiserum containing antibodies against the non-phosphorylated epitope ( $\alpha$ S364, top right). Underneath each blot the level of E2F-1 determined by immunoblotting with antibody KH-95 is shown. The E2F-1 polypeptide is shown.
- e) MCF7 cells were treated as described in a). For immunoprecipitation of E2F-1, cell extract was incubated with anti-E2F-1 antibody (KH-95) bound to protein A-beads. Phosphorylation of endogenous E2F-1 on Ser364 was monitored by immunoblotting with the phospho-specific antibody P-S364 (upper panel). The level of immunoprecipitated E2F-1 is shown (middle panel) and input E2F-1 protein is shown below (bottom panel).

a)

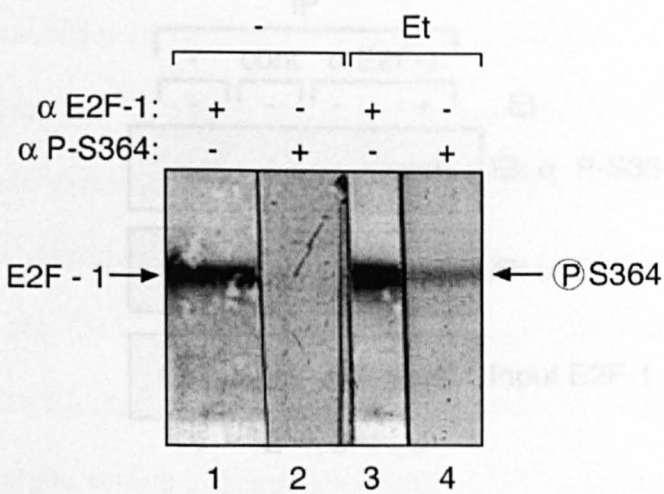




b)



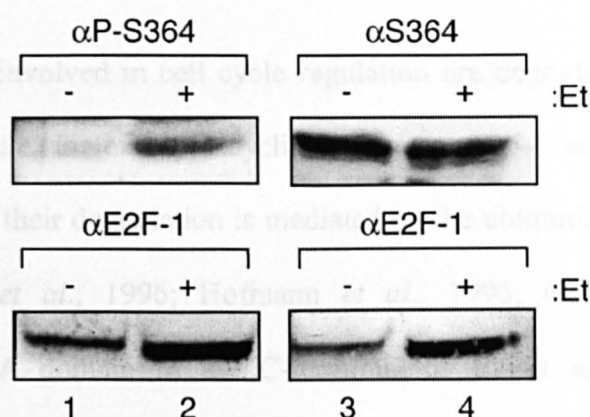
c)



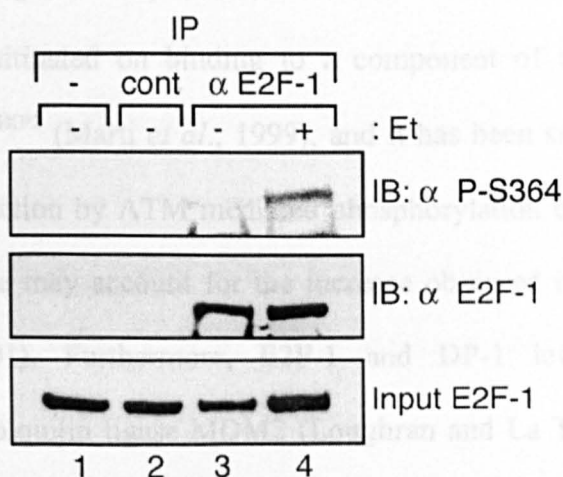
## Chapter 5. Regulation of E2F-1 stability

### 5.1. Introduction

d)



e)



Data presented in the previous chapters revealed that stabilization of E2F-1 in response to DNA damage is regulated by both ATM and Chk2 kinases. In addition, E2F-1 is phosphorylated on Ser364 by Chk2 *in vitro*, and on the same site *in vivo* in response to DNA damage. In this chapter the S364A mutant



# Chapter 5. Regulation of E2F-1 stability

## 5.1. Introduction

Many proteins involved in cell cycle regulation are degraded at defined points in the cell cycle, for example cyclins and cdis. E2F-1 and E2F-4 are unstable proteins and their degradation is mediated by the ubiquitin-proteasome pathway (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996; Campenero and Flemington, 1997). A domain in the C-terminus of E2F-1 and E2F-4 is responsible for their instability, and pocket protein binding masks the domain and stabilises both E2F-1 and E2F-4 (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996; Campenero and Flemington, 1997).

E2F-1 becomes ubiquitinated on binding to a component of the SCF ubiquitin-protein ligase p45<sup>SKP2</sup> (Marti *et al.*, 1999), and it has been suggested that disruption of this interaction by ATM mediated phosphorylation of E2F-1 in response to DNA damage may account for the increase observed in E2F-1 stability (Lin *et al.*, 2001). Furthermore, E2F-1 and DP-1 levels are downregulated by the E3 ubiquitin ligase MDM2 (Loughran and La Thangue, 2000), and p19<sup>ARF</sup> targets certain E2F species, including E2F-1, for proteasomal degradation (Martelli *et al.*, 2001).

Data presented in the previous chapters revealed that stabilisation of E2F-1 in response to DNA damage is regulated by both ATM and Chk2 kinases. In addition, E2F-1 is phosphorylated on Ser364 by Chk2 *in vitro*, and on the same site *in vivo* in response to DNA damage. In this chapter the S364A mutant

derivative of E2F-1 was studied to explore the role of Ser364 in the stabilisation of E2F-1 in response to DNA damage.

## **Results**

### **5.2. The Chk2 phosphorylation site in E2F-1 is required for DNA damage induction**

E2F-1 is stabilised in response to etoposide treatment (Figure 3.3a). To explore whether Ser364 in E2F-1 is important for DNA damage induction, the level of exogenous HA-tagged E2F-1 and S364A were compared in MCF7 cells. Under similar conditions of etoposide treatment, western blot analysis with a HA-specific antibody revealed that the temporal accumulation of S364A was about three or four times less than wild-type E2F-1 at 6 h (Figure 5.1, lane 3, compare top and bottom). Some accumulation of the S364A mutant was observed, possibly reflecting the activities of ATM, which has been shown recently to regulate E2F-1 stability through Ser31 phosphorylation (Lin *et al.*, 2001). This result suggests that Ser364 is an important site, involved in the control of E2F-1 stability in response to DNA damage.

### **5.3. Chk2 regulates E2F-1 stability**

One possibility to explain the results on the regulation of E2F-1 by Chk2, was that Chk2 controls E2F-1 abundance through protein stability. To test this idea, the overall protein synthesis of cells was blocked by addition of cycloheximide, and the degradation of exogenous HA-E2F-1 or S364A was

monitored by western blot analysis with a HA-specific antibody in etoposide treated and non-treated cells. Whilst E2F-1 and the S364A mutant were similar in the absence of etoposide (compare figure 5.2a, lane 3, top with figure 5.2b, lane 3, top), E2F-1 was significantly more stable than the S364A mutant in the presence of etoposide (compare figure 5.2a, lane 3, upper bottom with figure 5.2b, lane 3, upper bottom). Based on several different experiments, in the presence of etoposide E2F-1 persisted in cells for longer than the S364A mutant. As an internal control, PCNA was studied which was not affected by etoposide treatment.

#### **5.4. E2F-1 interacts with Chk2 kinase *in vitro* and *in vivo***

Chk2 interacts physically with some of its substrates, for example p53, PML and BRCA1 (Falck *et al.*, 2001b; Yang *et al.*, 2002; Lee *et al.*, 2000). In the previous chapter it was shown that Chk2 phosphorylates E2F-1 *in vitro* and *in vivo* in response to DNA damage. In order to investigate whether Chk2 physically interacts with E2F-1, *in vitro* binding experiments were performed using purified GST-E2F-1 and S364A proteins with <sup>35</sup>S-labelled *in vitro* transcribed and translated (IVT) Chk2. Chk2 interacted with GST-E2F-1 and S364A with similar efficiency (Figure 5.3a, compare lanes 3 and 4, top), but not with a GST alone control (Figure 5.3a, lane 2, top). The binding efficiency was compared to (5 %) Chk2 input (Figure 5.3a, lane 1, top). These results confirm that Chk2 and E2F-1 efficiently form a complex *in vitro*, and suggest that Ser364 is unlikely to reside within the region that binds to Chk2. Whilst the *in vitro* binding assay shows that the interaction is direct, it does not discriminate

between the binding efficiency of E2F-1 and S364A with Chk2. Therefore, an immunoprecipitation assay was performed to determine whether E2F-1 and S364A have different affinities for Chk2. MCF7 cells were transiently transfected with either HA-tagged E2F-1 or S364A. The levels of E2F-1 and S364A proteins were equal in extracts from these cells as confirmed by western blotting (Figure 5.3b, compare lanes 2 and 3, bottom). The extracts were immunoprecipitated with an anti HA-monoclonal antibody, followed by immunoblotting with an anti Chk2 polyclonal antibody to assess whether E2F-1 or S364A formed a complex with endogenous Chk2. As control, non-transfected extract was precipitated with HA-monoclonal antibody. Consistent with the *in vitro* data, both E2F-1 and S364A co-immunoprecipitated Chk2 with equal efficiency (Figure 5.3b, compare lanes 5 and 6, top). Therefore, Ser364 is unlikely to regulate the ability of E2F-1 to interact with Chk2.

It does not always follow that an interaction that occurs readily *in vitro*, or in over-expression studies will also occur under physiological conditions. In order to explore whether Chk2 and E2F-1 interact in cells, endogenous co-immunoprecipitation experiments were performed. Extracts from MCF7 cells were immunoprecipitated with an anti-E2F-1 antibody, followed by immunoblotting with an anti-Chk2 antibody to assess whether endogenous E2F-1 and Chk2 formed a complex. As control, precipitating antibody was omitted, or a non-specific antibody was used. The levels of E2F-1 and Chk2 proteins were equal in extracts from these cells as confirmed by western blotting (Figure 5.3c, compare lanes 1-3, top and bottom). Consistent with the *in vitro* and over-expression data, Chk2 and E2F-1 readily interact in cells, as Chk2 was present in E2F-1 immunoprecipitates (Figure 5.3c, lane 7, top). To investigate whether

the interaction is regulated in response to DNA damage, the same immunoprecipitation was performed using etoposide treated MCF7 cell extract. As expected, the level of E2F-1 protein was significantly increased in response to etoposide treatment (Figure 5.3c, compare lanes 3 and 4, bottom). In contrast Chk2 protein levels were unaffected (Figure 5.3c, compare lanes 3 and 4, top). The amount of E2F-1 immunoprecipitated increased in response to etoposide treatment (Figure 5.3c, compare lanes 7 and 8, bottom) as did the amount of co-immunoprecipitated Chk2 (Figure 5.3c, compare lanes 7 and 8, top). These results confirm that Chk2 and E2F-1 can interact under physiological conditions, and suggest that DNA damage is not essential for complex formation.

## **5.5. Mutation of E2F-1 to S364A does not affect binding to pRb**

pRb has been shown to regulate the stability of E2F-1 by preventing its degradation by the ubiquitin-proteasome pathway (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996; Campennero and Flemington, 1997). It is possible that this function of pRb may be regulated, for instance in response to DNA damage. In order to investigate whether E2F-1 and S364A have different affinities for pRb, *in vitro* binding experiments were performed using purified GST-E2F-1 and S364A proteins with <sup>35</sup>S-labelled IVT pRb. pRb interacted with GST-E2F-1 and S364A with similar efficiency (Figure 5.4a, compare lanes 3 and 4, top), but not with a GST alone control (Figure 5.4a, lane 2, top). The binding efficiency was compared to (5 %) pRb input (Figure 5.4a, lane 1, top). As expected, these

results confirm that pRb and E2F-1 efficiently form a complex *in vitro*, and suggest that substitution of Ser364 with alanine does not affect binding to pRb. In an attempt to discriminate between the binding efficiency of E2F-1 and S364A with pRb, an immunoprecipitation assay was performed. T98G cells were transiently transfected with either HA-tagged E2F-1 or S364A. The levels of E2F-1 and S364A proteins were equal in extracts from these cells as confirmed by western blotting (Figure 5.4b, compare lanes 2 and 3, bottom). The extracts were immunoprecipitated with an anti HA-monoclonal antibody, followed by immunoblotting with an anti pRb monoclonal antibody to assess whether E2F-1 or S364A formed a complex with endogenous pRb. As control, non-transfected extract was precipitated with HA-monoclonal antibody. Consistent with the *in vitro* data, both E2F-1 and S364A co-immunoprecipitated pRb (Figure 5.4b, compare lanes 5 and 6, top and bottom). To investigate whether the interaction is regulated in response to DNA damage, endogenous co-immunoprecipitation experiments were performed using etoposide treated or non-stressed T98G cell extract. As expected, the level of E2F-1 protein was significantly increased in response to etoposide treatment (Figure 5.4c, compare lane 2 and 3, bottom). In contrast pRb protein levels were unaffected (Figure 5.4c, compare lane 2 and 3, top). The amount of E2F-1 immunoprecipitated increased in response to etoposide treatment (Figure 5.4c, compare lanes 5 and 6, bottom) as did the amount of co-immunoprecipitated pRb (Figure 5.4c, compare lanes 5 and 6, top). Taken together, these results suggest that pRb binds to both E2F-1 and S364A, and under DNA damage conditions, induced E2F-1 in the cell is in complex with pRb.

## 5.6. Conclusions

The previous chapters established that the increase in E2F-1 accumulation observed in response to DNA damage is dependent on active Chk2, and that Chk2 phosphorylates Ser364 on E2F-1 in response to DNA damage. Work in this chapter has combined these two aspects to show that phosphorylation of Ser364 is required for stabilisation of E2F-1 in response to DNA damage.

Evidence was presented that Chk2 and E2F-1 are capable of forming a complex, and the interaction is likely to be direct as determined *in vitro*. Furthermore, Chk2 and E2F-1 interact in cells, signifying a potential physiological role for Chk2 in the regulation of E2F-1. These results suggest that Chk2 may regulate E2F-1 via two distinct mechanisms, by phosphorylating Ser364, and through their physical association.

In normal cells E2F activity is tightly regulated during the cell cycle through the temporal association and release of pocket proteins, and the binding of pRb to a domain of E2F-1 and E2F-4 has been shown to prevent their degradation by the ubiquitin-proteasome pathway. The possibility that pRb is involved in the stabilisation of E2F-1 in response to DNA damage, and that it may be mediated via Ser364 was also investigated. The results show that E2F-1 and S364A interact with pRb *in vitro* and in cells, and suggest that substitution of Ser364 with alanine does not affect the pRb-E2F-1 interaction. In addition, the amount of endogenous E2F-1 and pRb immunoprecipitated from cells increased in response to etoposide treatment. This suggests that induced E2F-1 in the cell is in complex with pRb, and likely reflects the hypo-phosphorylation

of pRb that has been observed in response to DNA damage (Ameltem *et al.*, 1996; Krtolica *et al.*, 1998).

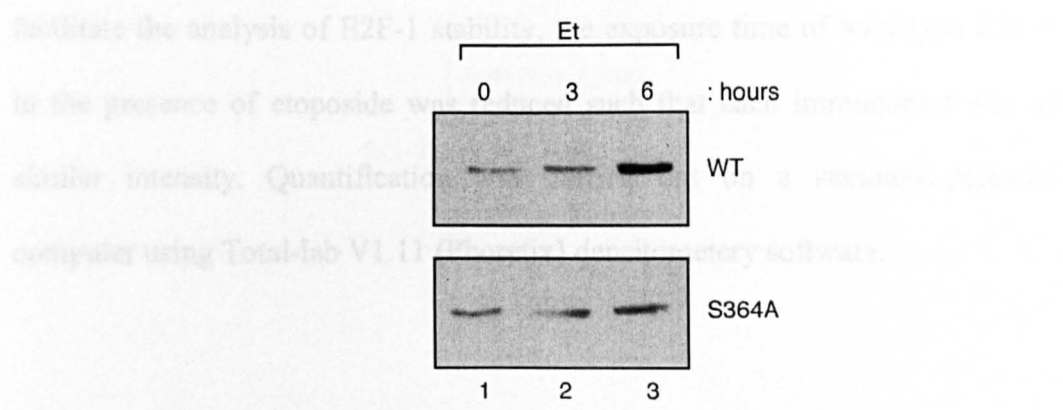


### **Figure 5.1. Dependence on Ser364 for E2F-1 accumulation**

- a) MCF7 cells were transfected with 500ng HA-E2F-1 or HA-S364A along with CMV- $\beta$ -gal (500ng) to monitor transfection efficiency, and then treated with etoposide (10 $\mu$ M) for the indicated time. Extracts were resolved by denaturing gel electrophoresis and HA-tagged E2F proteins were detected by immunoblotting with an HA-specific antibody. Quantification was carried out on a standard personal computer using Total-lab V1.11 (Phoretix) densitometry software.

Figure 5.2. Chk2 regulates E2F-1 stability

a) and b) U2OS cells were transfected with HA-E2F-1 (a) or HA-S364A E2F-1 (b) and treated with CMV-DPI, and treated with or without etoposide ( $10\mu\text{M}$ ) for a time indicated. Cycloheximide ( $50\mu\text{g}$ ) was then added and extracts prepared at the indicated times for HA-tagged E2F-1 or HA-S364A at the indicated times after cycloheximide addition. The level of PCNA is shown underneath each blot as a loading control.



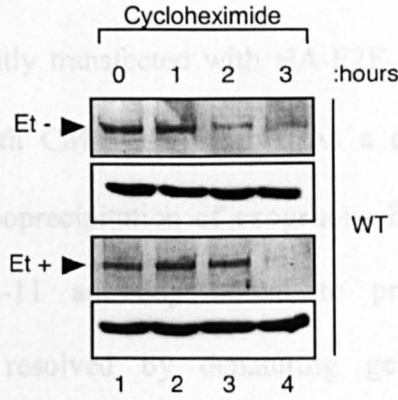
## **Figure 5.2. Chk2 regulates E2F-1 stability**

a) and b) U2OS cells were transfected with HA-E2F-1 (a) or HA-S364A (b) along with CMV-DP1, and treated with or without etoposide (10 $\mu$ M) for 4 h as indicated. Cycloheximide (50 $\mu$ g) was then added and extracts prepared and resolved for HA-tagged E2F-1 or HA-S364A at the indicated times by immunoblotting. The level of PCNA is shown underneath each treatment. To facilitate the analysis of E2F-1 stability, the exposure time of wild-type E2F-1 in the presence of etoposide was reduced such that each immunoblot was of similar intensity. Quantification was carried out on a standard personal computer using Total-lab V1.11 (Phoretix) densitometry software.

Figure 5.3. E2F-1 interacts with Chk2 *in vitro* and *in vivo*

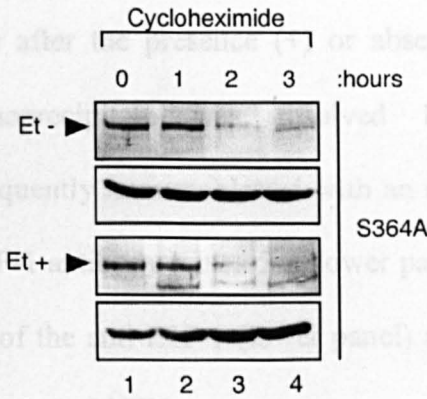
a) *In vitro* translated Chk2 was incubated with glutathione beads bound by or alone, GST-E2F-1 or GST-S364A. After extensive washing of the beads, proteins were released by addition of 1x SDS loading buffer and resolved by denaturing gel electrophoresis. Proteins were visualised by autoradiography.

a)



b) MCF7 cells were transiently transfected with pCMV-E2F-1 (5µg) or pCMV-S364A (5µg) in combination with Chk2 (5µg) or empty vector. Cells were treated with etoposide (5µg/ml) for 24 hours. Cell extracts were immunoprecipitated with anti-E2F-1 antibody and incubated with anti-PA-11. The immunoprecipitate was resolved by denaturing gel electrophoresis and subsequently immunoblotted with anti-Chk2, or anti-E2F-1 antibodies.

b)

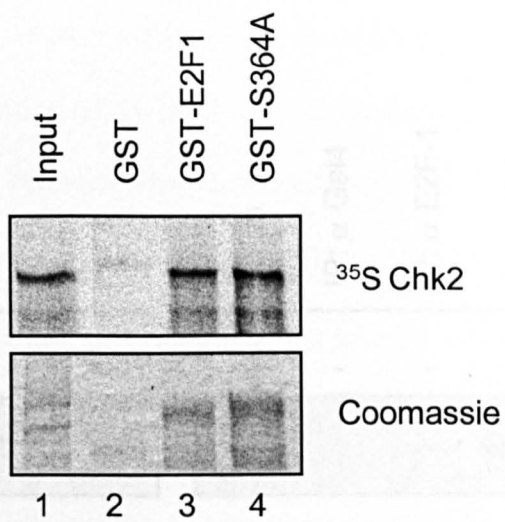


c) Extracts from asynchronously growing MCF7 cells were immunoprecipitated with anti-E2F-1 antibody after the presence (+) or absence (-) of etoposide treatment. The immunoprecipitate was resolved by denaturing gel electrophoresis and subsequently immunoblotted with anti-Chk2 (lanes 5-8, upper panel), and anti-E2F-1 (lanes 9-12, lower panel). Lanes 1-4 show the input (5%) reactivity of the antibodies with the crude MCF7 extract.

**Figure 5.3. E2F-1 interacts with Chk2 *in vitro* and *in vivo***

- a) *In vitro* translated Chk2 was incubated with glutathione beads bound to GST alone, GST-E2F-1 or GST-S364A. After extensive washing of the beads, proteins were released by addition of 1x SDS loading buffer and resolved by denaturing gel electrophoresis. Proteins were visualised by autoradiography.
- b) MCF7 cells were transiently transfected with HA-E2F-1 (5µg) or HA-S364A (5µg) in combination with CMV-DP-1 (1µg). As a control cells were left untransfected. For immunoprecipitation of exogenous E2F-1, cell extract was incubated with anti-HA-11 antibody bound to protein A beads. The immunoprecipitate was resolved by denaturing gel electrophoresis and subsequently immunoblotted with anti-Chk2, or anti-E2F-1 antibodies.
- c) Extracts from asynchronously growing MCF7 cells were immunoprecipitated with anti-E2F-1 antibody after the presence (+) or absence (-) of etoposide treatment. The immunoprecipitate was resolved by denaturing gel electrophoresis and subsequently immunoblotted with an anti-Chk2 (lanes 5-8, upper panel), and anti-E2F-1 antibody (lanes 5-8, lower panel). Lanes 1-4 show the input (5%) reactivity of the anti-E2F-1 (lower panel) and anti-Chk2 (upper panel) antibodies with the crude MCF7 extract.

a)



b)

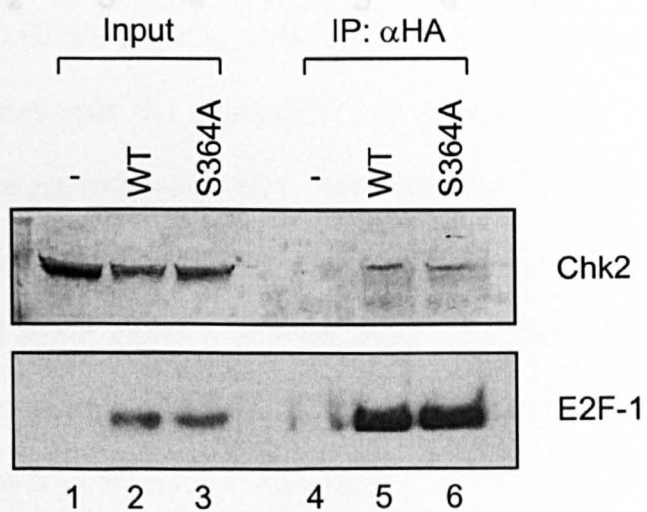
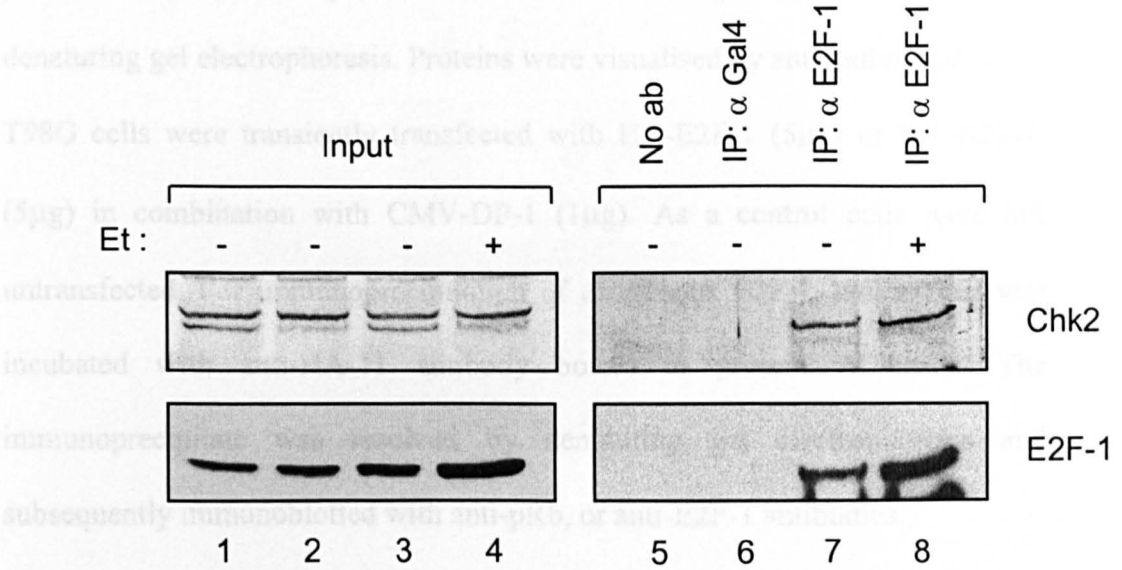


Figure 5.4. Ser364 does not regulate binding to pRb

*In vitro* translated pRb was incubated with glutathione beads bearing GST alone, GST-E2F-1 or GST-S364A. After extensive washing of beads, proteins were released by addition of 1x SDS loading buffer and separated by denaturing gel electrophoresis. Proteins were visualised by autoradiography.

c)



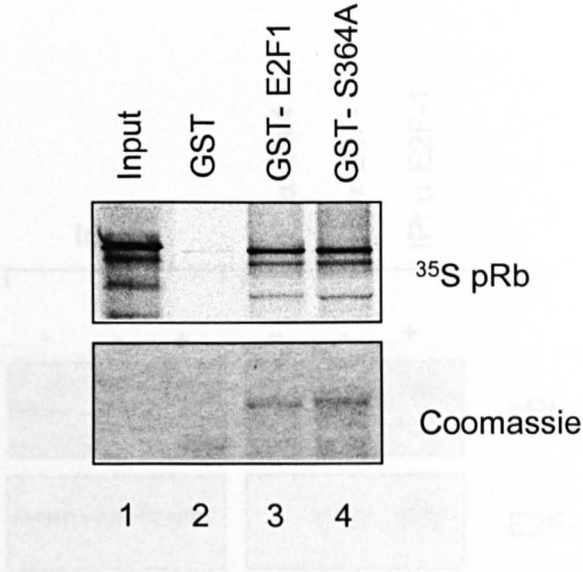
Extracts from asynchronously growing T98G cells were immunoprecipitated with anti-E2F-1 antibody after the presence (+) or absence (-) of etoposide treatment. The immunoprecipitate was resolved by denaturing gel electrophoresis and subsequently immunoblotted with anti-pRb (lanes 1-3, upper panel), and anti E2F-1 (lanes 4-6, lower panel) antibodies. Tracks 1-3 show the input (5%) reactivity of the anti-E2F-1 (lower panel) and anti-pRb (upper panel) antibodies with the crude T98G extract.

#### **Figure 5.4. Ser364 does not regulate binding to pRb**

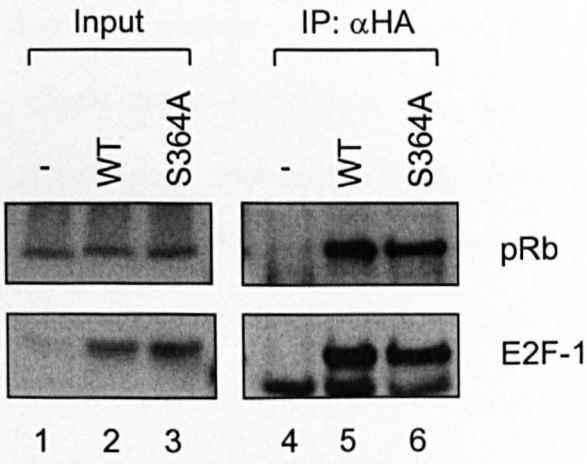
- a) *In vitro* translated pRb was incubated with glutathione beads bound to GST alone, GST-E2F-1 or GST-S364A. After extensive washing of the beads proteins were released by addition of 1x SDS loading buffer and resolved by denaturing gel electrophoresis. Proteins were visualised by autoradiography.
- b) T98G cells were transiently transfected with HA-E2F-1 (5µg) or HA-S364A (5µg) in combination with CMV-DP-1 (1µg). As a control cells were left untransfected. For immunoprecipitation of exogenous E2F-1, cell extract was incubated with anti-HA-11 antibody bound to protein A beads. The immunoprecipitate was resolved by denaturing gel electrophoresis and subsequently immunoblotted with anti-pRb, or anti-E2F-1 antibodies.
- c) Extracts from asynchronously growing T98G cells were immunoprecipitated with anti-E2F-1 antibody after the presence (+) or absence (-) of etoposide treatment. The immunoprecipitate was resolved by denaturing gel electrophoresis and subsequently immunoblotted with an anti-pRb (lanes 4-6, upper panel), and anti E2F-1 (lanes 4-6, lower panel) antibodies. Tracks 1-3 show the input (5%) reactivity of the anti-E2F-1 (lower panel) and anti-pRb (upper panel) antibodies with the crude T98G extract.



a)



b)

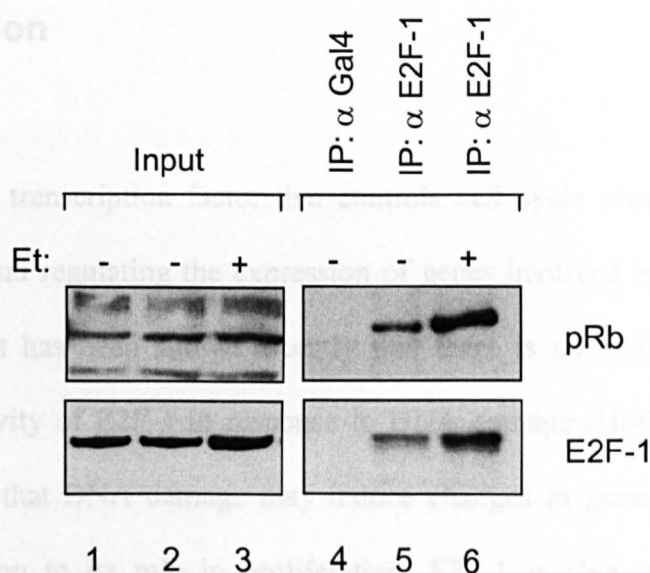


## Chk2

c)

## 6.1. Introduction

E2F-1 is a transcription factor involved in cell cycle progression. It binds to DNA and regulates the expression of genes involved in the G<sub>1</sub>/S phase transition. It has been shown that E2F-1 is a transcription factor with DNA-binding activity (1999), suggesting that it is involved in the regulation of gene expression profiles. In addition to its role in proliferation, E2F-1 is able to stimulate apoptosis, and thus possesses the properties of both an oncogene and tumor suppressor. E2F-1 can trigger apoptosis through both p53-dependent and independent pathways, and although transcription-independent functions of E2F-1 have been ascribed to apoptosis (Hsieh *et al.*, 1997; Phillips *et al.*, 1999; Phillips *et al.*, 1999), a number of genes involved in apoptosis are regulated at the transcriptional level by E2F-1 (Bates *et al.*, 1998; Morini *et al.*, 2001; Ivorra *et al.*, 2000; Sclowe *et al.*, 2001). Interestingly, it has also been reported that DNA damage induced E2F-1 is transcriptionally inactive (O'Connor *et al.*, 2000). In this chapter the functional consequence of phosphorylation of the Ser364 site in E2F-1 with respect to DNA binding, transcriptional activity, and apoptosis are explored.



# Chapter 6. Regulation of E2F-1 activity by Chk2

## 6.1. Introduction

E2F-1 is a transcription factor that controls cell cycle progression by binding to DNA and regulating the expression of genes involved in the G1/S-phase transition. It has been shown recently that there is an increase in the DNA-binding activity of E2F-1 in response to DNA damage (Hofferer *et al.*, 1999), suggesting that DNA damage may induce changes in gene expression profiles. In addition to its role in proliferation, E2F-1 is able to stimulate apoptosis, and thus possesses the properties of both an oncogene and tumour suppressor. E2F-1 can trigger apoptosis through both p53-dependent and independent pathways, and although transcription-independent functions of E2F-1 have been ascribed to apoptosis (Hsieh *et al.*, 1997; Phillips *et al.*, 1997; Phillips *et al.*, 1999), a number of genes involved in apoptosis are regulated at the transcriptional level by E2F-1 (Bates *et al.*, 1998; Moroni *et al.*, 2001; Irwin *et al.*, 2000; Stiewe *et al.*, 2001). Interestingly, it has also been reported that DNA damage induced E2F-1 is transcriptionally inactive (O'Connor *et al.*, 2000). In this chapter the functional consequence of phosphorylation of the Ser364 site in E2F-1 with respect to DNA-binding, transcriptional activation, and apoptosis are explored.

## **Results**

### **6.2. Mutation of E2F-1 to S364A does not affect heterodimer formation or DNA-binding**

The E2F family of proteins bind to DNA and regulate the transcription of target genes as part of an active heterodimer with a member of the DP family (Bandara *et al.*, 1993). To determine the functional consequences of E2F-1 phosphorylation at Ser364, the mutant derivative S364A was assessed in its ability to bind to DNA in association with DP-1. First, the ability of E2F-1 or S364A to dimerise with DP-1, and thus have the ability to efficiently bind DNA was tested. *In vitro* binding assays using purified GST-tagged E2F-1 proteins and <sup>35</sup>S-labelled IVT DP-1 revealed no difference between E2F-1 and S364A in their ability to bind to and form heterodimers with DP-1 (Figure 6.1a, compare lanes 3 and 4, top).

Next the DNA binding properties of GST-E2F-1 and S364A were examined *in vitro*. Gel retardation analysis using an E2A probe that contains E2F binding sites revealed that E2F-1 or S364A in combination with IVT DP-1 had a similar level of DNA-binding activity (Figure 6.1b, compare lanes 1-4 and 5-8).

Finally U2OS cells were left untransfected as control, or were transfected with HA-E2F-1 or S364A, in combination with DP-1. Gel retardation analysis with the E2A probe revealed that extracts from cells transfected with E2F-1 or S364A had a similar level of DNA-binding activity (Figure 6.1c, compare lanes 2 and 5). It has been reported that E2F-1 DNA

binding activity increases in response to IR (Hofferer *et al.*, 1999). However, when cells were DNA damaged by the addition of etoposide, E2F-1 and S364A exhibited a similar level of DNA binding that was unchanged from non-stressed cells (Figure 6.1c, compare lanes 8 and 11). Specificity was confirmed by shifting E2A bound complexes with E2F-1 (Figure 6.1c, lanes 3, 6, 9, and 12) or DP-1 antibodies (Figure 6.1c, lanes 4, 7, 10 and 13). The results suggest that substitution of Ser364 with alanine does not affect the ability of E2F-1 to heterodimerise with DP-1 or bind to DNA.

### **6.3. S364A has a compromised transcriptional activity**

The functional importance of the Ser364 site for E2F-1 dependent transcription was assessed by comparing the properties of E2F-1 and S364A on E2F-responsive promoters. For this, several promoters were employed including the cyclinE, ARF and Apaf-1 promoters, which are directly responsive to E2F-1 (Botz *et al.*, 1996, Bates *et al.*, 1998., Moroni *et al.*, 2001). U2OS cells were transiently transfected with a cyclinE-luciferase reporter gene and expression vectors for E2F-1 or S364A in combination with DP-1. Consistent with reports that E2F and DP bind synergistically to the E2F site in target genes (Dyson, 1998), transfection of DP-1 in the absence of E2F-1 resulted in an insignificant stimulation of reporter activity (Figure 6.2a, lane 2). Co-transfection of E2F-1 with DP-1 resulted in a significant induction of reporter assay activity (Figure 6.2a, lane 3), and titration of E2F-1 in the presence of a constant amount of DP-1 induced reporter activity in a dose-dependent manner (Figure 6.2a, lanes 3-6). Co-transfection of S364A with DP-1

revealed that the activity of S364A was significantly lower than E2F-1 (Figure 6.2a, lanes 7-10), even though the protein levels of S364A and E2F-1 were similar (Figure 6.2a, bottom panel). These observations imply that S364A has a compromised transcriptional activity.

It has been demonstrated that the p19<sup>ARF</sup> promoter is sensitive to E2F-1 over-expression (Bates *et al.*, 1998), therefore a p19<sup>ARF</sup>-luciferase reporter that consists of the exon1 $\beta$  promoter region, which contains four potential E2F consensus sites was employed (Bates *et al.*, 1998). Consistent with the results using the cyclinE-luciferase reporter gene, U2OS cells transfected with exon1 $\beta$ -luciferase and E2F-1 or S364A in combination with DP-1 revealed that the activity of the S364A mutant was compromised compared to E2F-1 (Figure 6.2b compare lanes 3-6 with 7-10). In order to see whether an increase in E2F-1 transcriptional activity could be observed in response to DNA damage, U2OS cells were transfected as before and treated with etoposide for 16 h prior to harvesting. No increase in either E2F-1 or S364A activity was observed in response to etoposide treatment (Figure 6.2b, lanes 11-14 and 15-18). Conversely, there was a decrease in the level of E2F-dependent transcription.

Recent studies have identified the gene for the apoptosis promoting factor Apaf-1 as a transcriptional target of E2F-1 (Moroni *et al.*, 2001). The Apaf-1 promoter can be activated by both E2F-1 and p53 (Moroni *et al.*, 2001), therefore an Apaf-1 reporter construct (-396/+208), which contains only E2F responsive elements was used to rule out any contribution from endogenous p53 (Moroni *et al.*, 2001). In U2OS cells, transiently transfected with Apaf-1(-396/+208)-luciferase and E2F-1 or S364A in combination with DP-1, once

again the activity of the S364A mutant was compromised compared to E2F-1 (Figure 6.2c, compare lanes 3-6 and 7-10).

It has been reported that Chk2 is required for p53-dependent transcription in mouse cells (Takai *et al.*, 2002), and Chk2 dominant-negative can down-regulate both p53 and E2F-1 at the protein level in response to etoposide treatment (Figure 3.5a). Therefore, a Chk2 dominant-negative mutant (Asp347Ala) was employed to assess if Chk2 could influence the ability of E2F-1 to transactivate the Apaf-1 promoter. U2OS cells were transfected with Apaf-1(-396/+208)-luciferase and fixed amounts of E2F-1 in combination with DP-1. Titration of Chk2 dominant-negative in the presence of E2F-1 and DP-1 yielded no change in transcriptional activation of the Apaf-1-luciferase reporter gene (Figure 6.2d, lanes 4-6). Data presented previously showed that expression of Chk2 dominant-negative in the absence of etoposide treatment had no effect on E2F-1 protein levels, however in the presence of etoposide treatment a significant down-regulation of E2F-1 protein was observed (Figure 3.5a). Therefore, U2OS cells transfected as described before were treated for 6 h with etoposide prior to harvesting. No change in E2F-1 dependent transcription was observed when Chk2 dominant-negative was over-expressed in the presence of etoposide treatment (Figure 6.2d, lanes 10-12).

In a manner similar to the previous experiment, U2OS cells were transiently transfected with Apaf-1(-396/+208)-luciferase and fixed amounts of E2F-1 in combination with DP-1. Titration of Chk2 wild-type in the presence of E2F-1 and DP-1 yielded no change in the transcriptional activation of the Apaf-1-luciferase reporter gene (Figure 6.2e, lanes 4-6). Exogenous Chk2 is Thr68 phosphorylated and activated after 6 h of etoposide treatment (Figure 3.5d),

however no effect on E2F-1 transcriptional activity was observed when Chk2 wild-type was over-expressed under similar conditions of etoposide treatment (Figure 6.2e lanes 10-12).

These results suggest that Ser364 is a functionally important site that contributes to the ability of E2F-1 to activate transcription, however no direct effect of Chk2 on E2F-1 dependent transcription was observed.

## **6.4. Chk2 regulates E2F-1-dependent apoptosis**

Over-expression of E2F-1 can induce apoptosis (Degregori *et al.*, 1997; Loughran and La Thangue, 2000). If Chk2 is involved in regulating E2F-1-dependent apoptosis, then dominant-negative Chk2 should interfere with E2F-1 apoptotic activity. Evidence for a role for Chk2 in the control of E2F-1 activity came from comparing E2F-1 to S364A, where in U2OS cells apoptosis induced by E2F-1 was considerably greater than observed with S364A (Figure 6.3a). Moreover, whilst the co-expression of the Chk2 dominant-negative with E2F-1 reduced the level of apoptosis, this was not apparent with apoptosis induced by S364A (Figure 6.3a).

In order to rule out a contribution to apoptotic activity from p53, apoptosis in SAOS2 cells ( $p53^{-/-}$ ) was investigated. As in U2OS cells, E2F-1 possessed greater apoptotic activity than S364A, and co-expression of the Chk2 dominant-negative reduced the apoptotic activity of E2F-1, but failed to significantly affect the activity of S364A (Figure 6.3b). These results support the notion that Chk2 regulates E2F-1-dependent apoptosis in response to DNA damage.



## 6.5. Induction of E2F-1 correlates with the induction of genes involved in apoptosis

E2F-1 can induce apoptosis independently of p53, and E2F-1 responsive genes involved in apoptosis include p73, Apaf-1 and ARF (Irwin *et al.*, 2000; Moroni *et al.*, 2001; Bates *et al.*, 1999). To investigate the mechanism through which E2F-1 induces apoptosis in response to DNA damage, SAOS2 cells (*p53*<sup>-/-</sup>) were stimulated to enter apoptosis by treatment with etoposide. Western blot analysis of cell extracts revealed that the induction of endogenous E2F-1 correlated well with the induction of Apaf-1, p73 and ARF (Figure 6.4a, b, and c). These results support a role, independent of p53, for E2F-1 in etoposide-induced apoptosis via the transcriptional activation of E2F-1 target genes such as p73, Apaf-1 and ARF.

## 6.6. Conclusions

E2F-1 must form a heterodimer with DP-1 to efficiently bind DNA and activate, or repress transcription of E2F target genes (Dyson, 1998). To gain insight into the functional consequence of E2F-1 phosphorylation by Chk2, DNA-binding activity was examined. *In vitro* binding assays showed that mutation of Ser364 to alanine has no significant effect on the ability of E2F-1 to dimerise with DP-1 or bind to DNA. Moreover, transfection based experiments revealed no difference in E2F-1 or S364A DNA-binding activity in cells under non-stress or stress conditions.

Transcription based assays using various reporter genes established that Ser364 is an important site for E2F-1-dependent transcription. The activity of the S364A mutant derivative was reproducibly lower than E2F-1 under non-stress conditions. Treatment of cells with etoposide failed to stimulate E2F-1 or S364A transcriptional activity, therefore the compromised S364A activity may be due to altered binding to a cellular co-factor, such as p300/CBP which has been previously shown to co-activate E2F-1 transcription (Trouche *et al.*, 1996). Finally, over-expression of Chk2 or a Chk2 dominant-negative derivative was unable to significantly alter E2F-1 transcriptional activity, even when cells were stressed by treatment with etoposide.

E2F-1 can induce apoptosis independently of p53. In SAOS2 cells ( $p53^{-/-}$ ), it was shown that S364A has a compromised ability to induce apoptosis, suggesting that phosphorylation by Chk2 may regulate E2F-1 dependent apoptosis through the Ser364 site. Further evidence in support of this idea came from the observation that Chk2 dominant-negative can overcome E2F-1 but not S364A mediated apoptosis.

The observed effects on apoptosis were linked to E2F-1-dependent transcription. In SAOS2 cells stimulated to enter apoptosis, the induction of endogenous E2F-1 correlated well with the induction of proteins encoded by E2F responsive genes involved in apoptosis, such as Apaf-1, p73 and ARF.

Data presented in this chapter support a role, independently of p53, for Chk2 in the regulation of E2F-1-dependent apoptosis through a transcription-

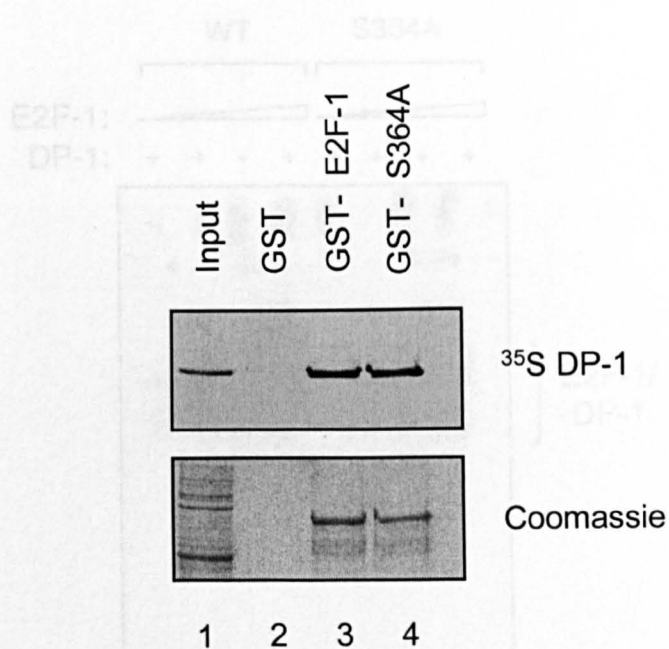
based mechanism.

### Figure 6.1. E2F-1 DNA-binding activity

- a) *In vitro* translated DP-1 was incubated with glutathione beads bound to GST alone (1µg), GST-E2F-1 (1µg) or GST-S364A (1µg). After extensive washing of the beads, bound proteins were released by addition of 1xSDS loading buffer and resolved by gel electrophoresis. Proteins were visualised by autoradiography.
- b) Gel retardation analysis of a mixture of recombinant E2F-1 (0.25, 0.5, 1, or 2µg) or S364A (0.25, 0.5, 1, or 2µg), with *in vitro* translated DP-1 (1µg). Complexes bound to <sup>32</sup>P labelled DNA were visualised by autoradiography.
- c) Gel retardation analysis of extracts from U2OS cells either untransfected or transfected with expression vectors for HA-tagged E2F-1 or HA-tagged S364A (5µg) and CMV-DP-1 (1µg) as indicated. Cells were left untreated as control or treated with 10µM etoposide for 6 h. Positions of the free E2F-1/DP-1 heterodimer, E2F-1/DP-1/pRb complex and the E2F-1/DP-1/pRb complex shifted with antibodies specific to E2F-1 or DP-1 are indicated. Complexes bound to <sup>32</sup>P labelled DNA were visualised by autoradiography.

b)

a)



b)

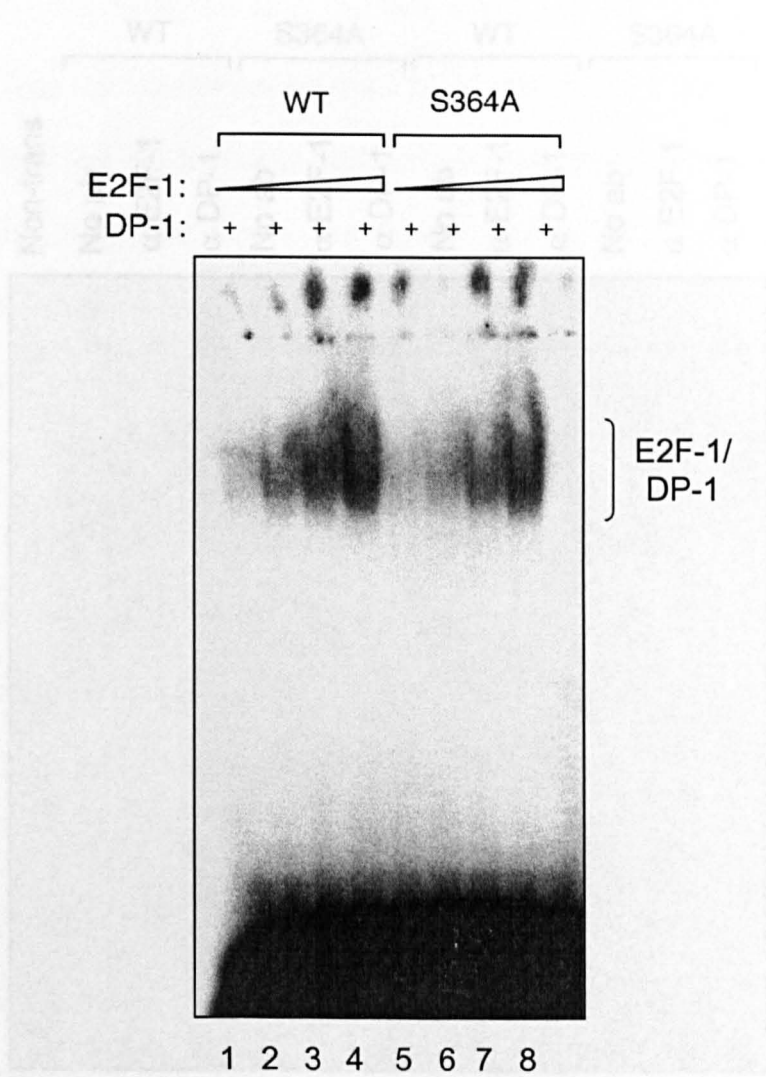
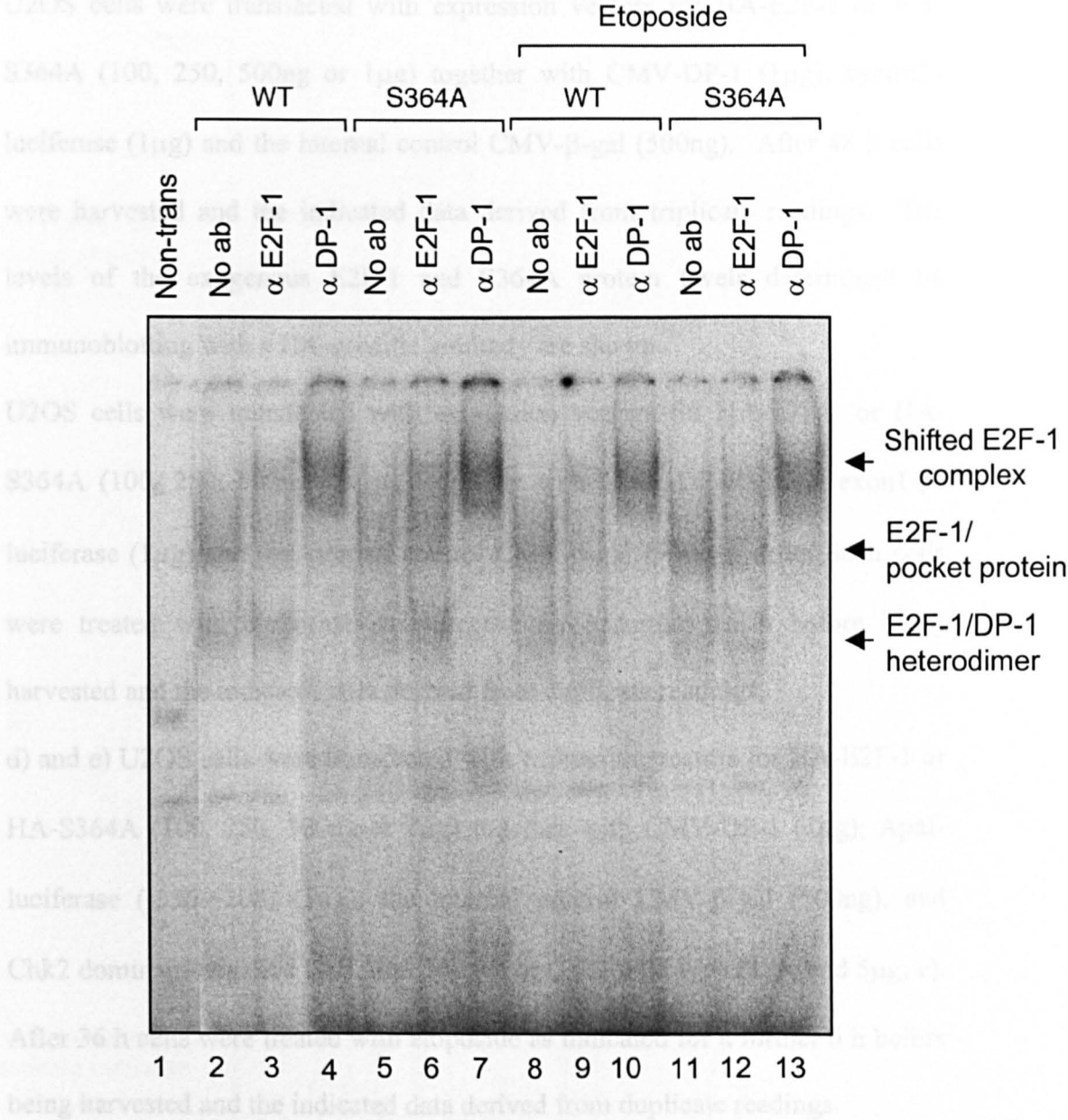


Figure 4.3. Ser364 regulates E2F-1 transcriptional activity

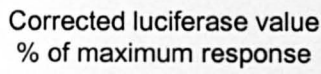
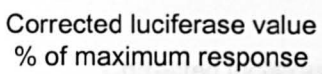
c)



## **Figure 6.2. Ser364 regulates E2F-1 transcriptional activity**

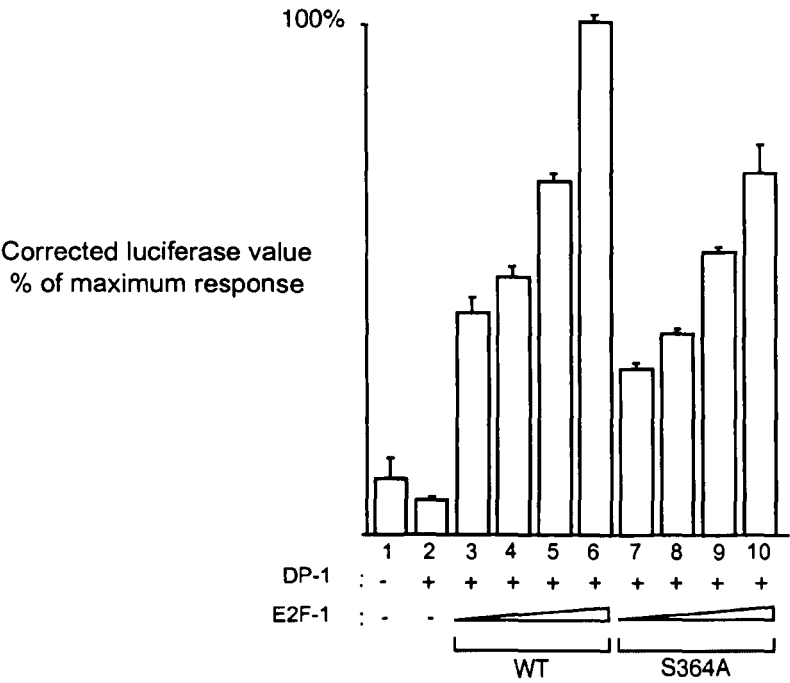
- a) U2OS cells were transfected with expression vectors for HA-E2F-1 or HA-S364A (100, 250, 500ng or 1 $\mu$ g) together with CMV-DP-1 (1 $\mu$ g), cyclinE-luciferase (1 $\mu$ g) and the internal control CMV- $\beta$ -gal (500ng). After 48 h cells were harvested and the indicated data derived from triplicate readings. The levels of the exogenous E2F-1 and S364A protein levels determined by immunoblotting with a HA-specific antibody are shown.
- b) U2OS cells were transfected with expression vectors for HA-E2F-1 or HA-S364A (100, 250, 500ng or 1 $\mu$ g) together with CMV-DP-1 (1 $\mu$ g), exon1- $\beta$ -luciferase (1 $\mu$ g) and the internal control CMV- $\beta$ -gal (500ng). After 30 h cells were treated with etoposide as indicated for a further 16 h before being harvested and the indicated data derived from duplicate readings.
- c) d) and e) U2OS cells were transfected with expression vectors for HA-E2F-1 or HA-S364A (100, 250, 500ng or 1 $\mu$ g) together with CMV-DP-1 (1 $\mu$ g), Apaf-luciferase (-396/+208) (1 $\mu$ g), the internal control CMV- $\beta$ -gal (500ng), and Chk2 dominant-negative (1, 3, and 5 $\mu$ g; d) or Chk2 wild-type (1, 3, and 5 $\mu$ g; e). After 36 h cells were treated with etoposide as indicated for a further 6 h before being harvested and the indicated data derived from duplicate readings.



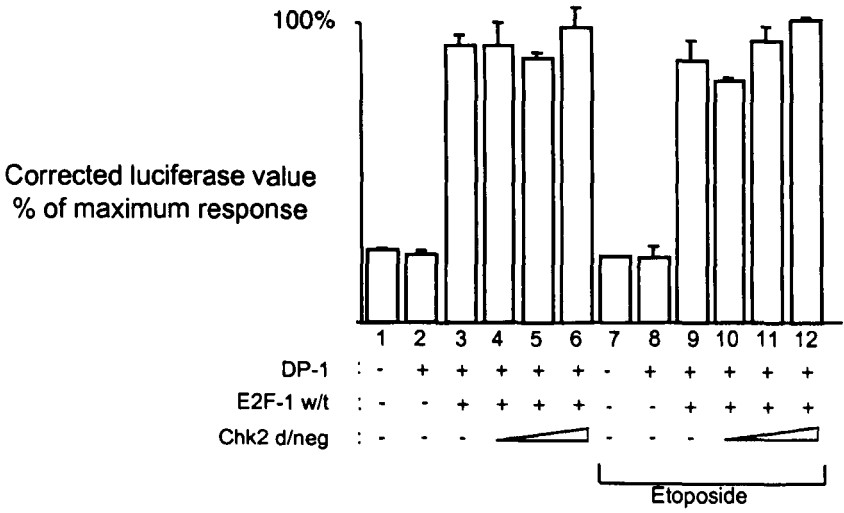


Etoposide

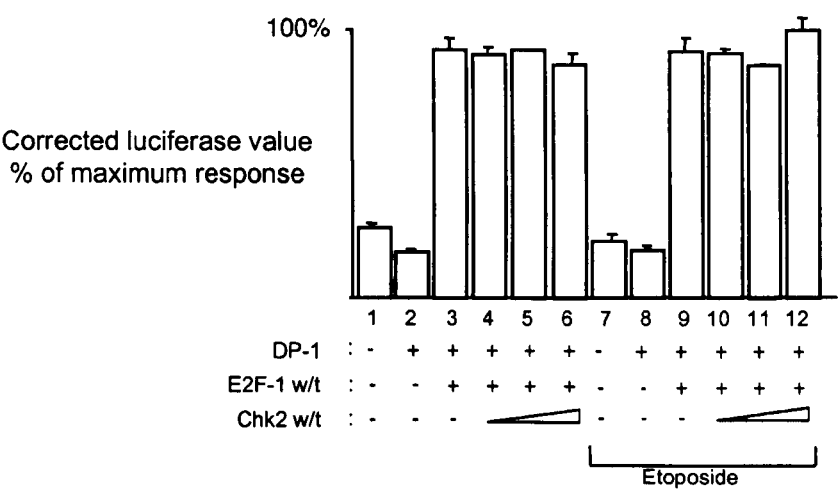
c)



d)



e)



### **Figure 6.3. Apoptosis and cell cycle arrest through E2F-1 requires Chk2**

- a) U2OS cells were transfected with vector for E2F-1 (5μg) or S364A (5μg) and DP-1 (5μg), together with dominant-negative Chk2 (10μg) as indicated, and treated as described above. The graph shows the percentage cells in the apoptosing (sub-G1 DNA content) population with respect to the vector alone treatment without etoposide (sub-G1 14.1%).
- b) SOAS2 (*p53*<sup>-/-</sup>) cells were transfected with vectors for E2F-1 (300ng) or S364A (300ng) and DP-1 (300ng) and together with dominant-negative Chk2 (10μg) treated as described with etoposide (20μM) for 6 h. The graph shows the percentage cells in the apoptosing (sub-G1 DNA content) population with respect to the vector alone treatment without etoposide (sub-G1 16.7%).

Figure 6.4. Pathways to E2F-1 mediated apoptosis

a) OS2 (p53<sup>-/-</sup>) cells were treated with etoposide (10μM) for 0, 2, 4, 8, and 16 h.

Extracts were prepared and immunoblotted for endogenous p21 (top), E2F-1 (middle) and PCNA (bottom).

(middle) and PCNA (bottom).

b) SAOS2 (p53<sup>-/-</sup>) cells were treated with etoposide (10μM) for 0, 4, and 16 h.

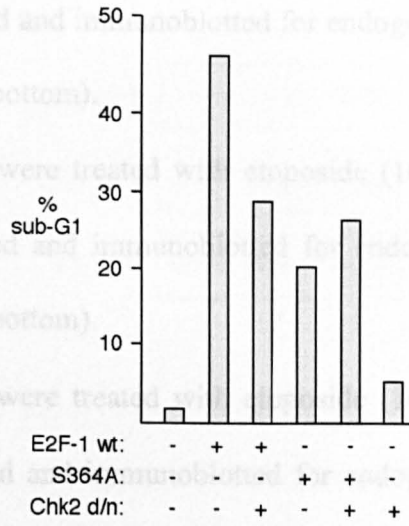
Extracts were prepared and immunoblotted for endogenous p21 (top), E2F-1 (middle) and PCNA (bottom).

(middle) and PCNA (bottom).

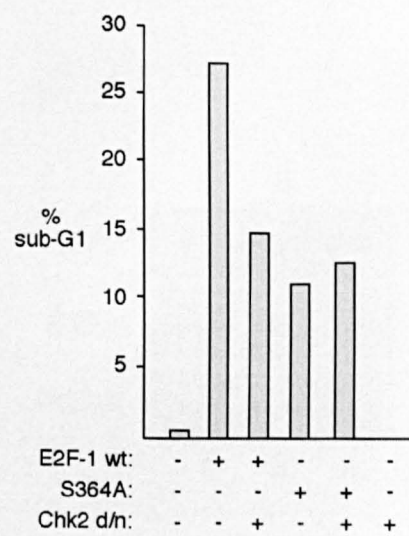
c) SAOS2 (p53<sup>-/-</sup>) cells were treated with etoposide (10μM) for 0, 4, and 16 h.

Extracts were prepared and immunoblotted for endogenous p21 (top), E2F-1 (middle) and PCNA (bottom).

(middle) and PCNA (bottom).



b)

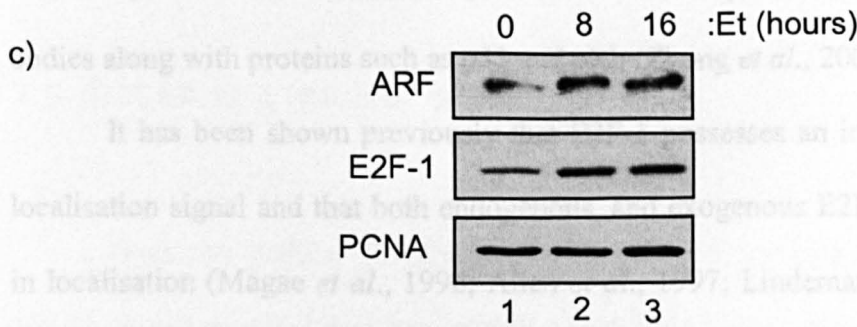
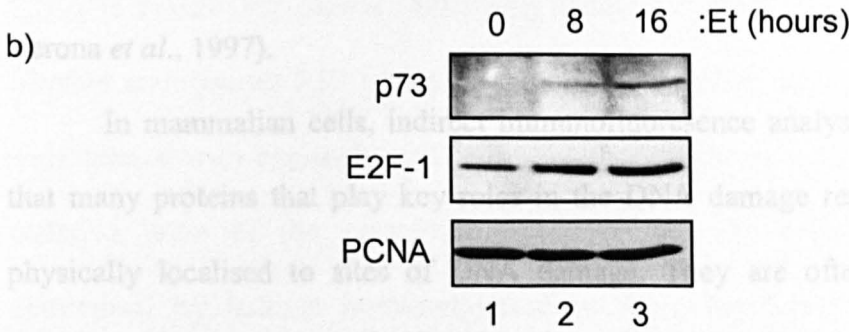
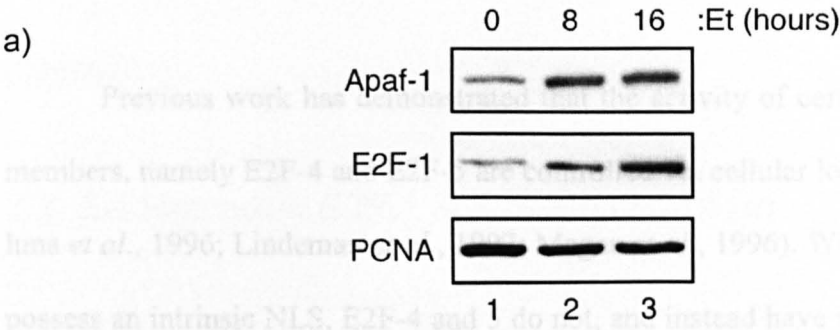


#### **Figure 6.4. Pathways to E2F-1 mediated apoptosis**

- a) SAOS2 (*p53*<sup>-/-</sup>) cells were treated with etoposide (10μM) for 0, 8, and 16 h. Extracts were prepared and immunoblotted for endogenous Apaf-1 (top), E2F-1 (middle) and PCNA (bottom).
- b) SAOS2 (*p53*<sup>-/-</sup>) cells were treated with etoposide (10μM) for 0, 8, and 16 h. Extracts were prepared and immunoblotted for endogenous p73 (top), E2F-1 (middle) and PCNA (bottom).
- c) SAOS2 (*p53*<sup>-/-</sup>) cells were treated with etoposide (10μM) for 0, 8, and 16 h. Extracts were prepared and immunoblotted for endogenous ARF (top), E2F-1 (middle) and PCNA (bottom).

# Chapter 7. Cellular localisation of E2F-1

## 7.1 Introduction



# Chapter 7. Cellular localisation of E2F-1

## 7.1 Introduction

Previous work has demonstrated that the activity of certain E2F family members, namely E2F-4 and E2F-5 are controlled via cellular localisation (de la luna *et al.*, 1996; Lindeman *et al.*, 1997; Magae *et al.*, 1996). Whereas E2Fs 1-3 possess an intrinsic NLS, E2F-4 and 5 do not, and instead have a NES (Gaubatz *et al.*, 2001). They can however be imported into the nucleus in association with a bound pocket protein or appropriate DP family member (Allen *et al.*, 1997; Verona *et al.*, 1997).

In mammalian cells, indirect immunofluorescence analyses have shown that many proteins that play key roles in the DNA damage response become physically localised to sites of DNA damage. They are often observed as brightly staining spots or “foci” within the cell nucleus. Such proteins include ATR, the Mre11/Rad50/Nbs1 complex, BRCA1, 53BP1 and the activated form of Chk2 (Rouse and Jackson, 2002). In addition PML protein localises to PML bodies along with proteins such as p53 and pRb (Zhong *et al.*, 2000).

It has been shown previously that E2F-1 possesses an inherent nuclear localisation signal and that both endogenous, and exogenous E2F-1 are nuclear in localisation (Magae *et al.*, 1996; Allen *et al.*, 1997; Lindeman *et al.*, 1997). The phosphorylation status of a protein can control its cellular localisation, for example export of Cdc25C from the nucleus by 14-3-3 proteins is dependent on the phosphorylation of Cdc25C by Chk kinases (Peng *et al.*, 1997). It was possible that phosphorylation of E2F-1 by Chk2, or the physical association of



E2F-1 with Chk2 could affect its ability to localise to the nucleus. The objectives of this chapter were to investigate whether Chk2 regulates E2F-1 activity by influencing its cellular localisation.

## **Results**

### **7.2. Endogenous E2F-1 is a nuclear protein**

First, the cellular location and staining pattern of endogenous E2F-1 was determined using the general E2F-1 antibody KH-95. As shown in (Figure 7.1a) E2F-1 is exclusively nuclear, exhibiting diffuse but even staining. To explore whether endogenous E2F-1 would be affected by DNA damage, MCF7 cells were treated with etoposide or UV-irradiation (which are believed to activate different arms of the signalling pathway), and the cellular distribution determined by indirect immunofluorescence using the KH-95 antibody. No significant change in distribution of the protein, or in its intensity of staining was observed with either treatment (Figure 7.1b and c).

### **7.3. S364A is a nuclear protein**

Next the cellular distribution of HA-tagged versions of E2F-1 and S364A were compared. HA-tagged E2F-1 and S364A were expressed in U2OS cells by transient transfection and their cellular distribution determined by indirect immunofluorescence. In MCF7 cells E2F-1 and S364A proteins

exhibited nuclear only staining with an HA-specific antibody (Fig. 7.2a and 7.3a).

To explore whether HA-E2F-1 or S364A would be affected by DNA damage, transiently transfected MCF7 cells were treated with UV-irradiation or etoposide prior to immunostaining. No change in the cellular localisation or staining intensity of E2F-1 or S364A was observed in response to UV treatment (Fig. 7.2b and 7.3b), or etoposide treatment (Fig. 7.2c and 7.3c).

Therefore the S364A mutant derivative did not differ from E2F-1 in its cellular localisation in the absence or presence of DNA damage, suggesting that phosphorylation of E2F-1 on Ser364 does not affect its cellular localisation.

#### **7.4. E2F-1 is unaffected by co-expression of Chk2**

Data presented in chapter 5 revealed that Chk2 can form a complex with E2F-1 *in vitro* and *in vivo*. It remained possible that Chk2 could regulate the intracellular localisation of E2F-1 through their direct binding. To explore this possibility U2OS cells were co-transfected with HA-E2F-1 and Flag-Chk2. As expected Chk2 is a nuclear protein and exhibited diffuse staining throughout the nucleus with a general Chk2 antibody (Figure 7.4 d). U2OS cells have low levels of endogenous Chk2 (Chehab *et al.*, 2000), therefore cells transfected with Flag-Chk2 were easily distinguishable with the Chk2 antibody (Figure 7.4, compare d with g). In cells that co-expressed E2F-1 and Chk2, no change in E2F-1 intensity or cellular localisation was observed (compare figures 7.2a and 7.4a). Since Chk2 is activated in response to DNA damage in an ATM-dependent manner (Matsuoka *et al.*, 1998), it was possible that Chk2 would

modulate E2F-1 localisation only under DNA damage conditions. To explore this idea, U2OS cells co-transfected with HA-E2F-1 and Flag-Chk2 were treated with UV-irradiation or etoposide prior to immunostaining. Under these conditions there was no obvious change in the intensity or cellular localisation of either E2F-1 (Figure 7.4, b and c) or Chk2 (Figure 7.4, e and f).

To explore whether Chk2 dominant-negative could regulate E2F-1 through altered cellular localisation, U2OS cells were co-transfected with HA-E2F-1 and Flag-Chk2 dominant-negative. As expected Chk2 dominant-negative exhibited diffuse staining throughout the nucleus with a general Chk2 antibody (Figure 7.5, d). Similar to the results observed with Chk2, co-expression of Chk2 dominant-negative had no effect on E2F-1 intensity, or cellular localisation (compare figures 7.2a and 7.5a).

Since Chk2 dominant-negative can block the induction of endogenous E2F-1 only when cells are exposed to etoposide treatment, it was possible that it would modulate E2F-1 localisation only under DNA damage conditions. To test this idea U2OS cells over-expressing HA-E2F-1 in combination with Flag-Chk2 dominant-negative were treated with UV-irradiation or etoposide prior to immunostaining. Under these conditions, there was no obvious change in the intensity or cellular localisation of either E2F-1 (Figure 7.5, b and c) or Chk2 dominant-negative (Figure 7.5, e and f). Therefore, Chk2 does not regulate the intracellular localisation of E2F-1.

## 7.5. E2F-1 phosphorylated on Ser364 resides in discrete nuclear structures

Some proteins, such as Chk2, that exhibit a diffuse nuclear staining with a general antibody, localise to discrete nuclear foci when activated and detected using a phosphorylation-specific antibody (Zhou and Elledge, 2000; Ward *et al.*, 2000). To study the location of E2F-1 protein phosphorylated on Ser364, the affinity purified P-S364 antibodies were used. In normal cells immunostaining with the affinity purified antibody produced a low level of diffuse but evenly distributed nuclear staining (Figure 7.6, a and b). The immunostaining pattern produced by P-S364 was specific since stained nuclei were competed only by inclusion of the phosphorylated Ser364 peptide and not by the unphosphorylated E2F-1 peptide (Figure 7.6, compare c and d), indicating that a low level of E2F-1 phosphorylated on Ser364 exists in non-stressed cells.

To explore whether the staining pattern would be affected by DNA damage, MCF7 cells were treated with etoposide before indirect immunofluorescence with the P-S364 antibody was performed. In etoposide treated cells, whilst the overall level of nuclear stain exhibited a moderate increase, the increase occurred in parallel with the appearance of discrete nuclear structures with intense staining (Figure 7.7, a and b). Again, this staining pattern was specific for E2F-1 phosphorylated on Ser364 since it was competed by the phosphorylated and not the unphosphorylated peptide (Figure 7.7, compare c and d). These results support the conclusion that the Chk2 phosphorylation of Ser364 is induced by etoposide treatment, and further imply

the existence of specific nuclear sub-structures in which the phosphorylated species of E2F-1 can reside.

## **7.6. E2F-1 co-localisation**

The sub-nuclear structures or foci observed in which P-S364 E2F-1 resides are large and not very numerous (Figure 7.7a and b). Other nuclear structures that occur with a similar frequency, and of a similar size within the nucleus include PML bodies (Lamond and Earnshaw, 1998). To check if P-S364 E2F-1 colocalises to PML bodies, SAOS2 cells were treated with etoposide over increasing time. Whilst P-S364 E2F-1 foci formed from 3 h and persisted to 16 h, they decreased in number and increased in size (Figure 7.8, a-d). Conversely, staining with a PML antibody was apparent in the absence of etoposide treatment and remained unchanged up to the 8 h timepoint, however after 16 h of etoposide treatment, a marked reduction in PML staining was observed (Figure 7.8, e-h). Importantly, merging of the P-S364 and PML fields revealed that no co-localisation of P-S364-E2F-1 and PML was observed under any conditions (Figure 7.8, i-l). Therefore phosphorylated Ser364 E2F-1 does not reside within PML bodies.

## **7.7. Conclusions**

In this chapter the cellular distribution of E2F-1 has been explored. Endogenous and exogenous E2F-1 localised to the nucleus, and no significant change in the intracellular distribution or intensity of staining was observed

when cells were DNA damaged by etoposide or UV-irradiation. In addition, the sub-cellular localisation of the mutant derivative S364A did not differ from E2F-1 under any of the conditions tested. Co-expression studies of E2F-1 with Chk2 (wild-type and dominant-negative) revealed no significant change in E2F-1 intensity or cellular localisation.

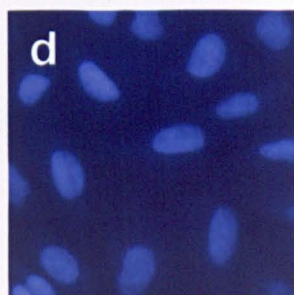
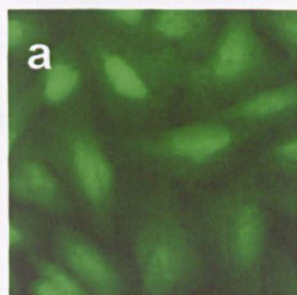
However, use of the P-S364 antiserum revealed that E2F-1 phosphorylated on Ser364 resides within discrete nuclear structures with intense staining, supporting the earlier conclusion that E2F-1 is phosphorylated by Chk2 in cells in response to DNA damage. Based upon their size and frequency within the nucleus, the possibility that these structures were PML bodies was assessed. However the results suggest that P-S364-E2F-1 is unlikely to localise to these sub-nuclear structures. Further investigation of the specific structures will be required to reveal their functional significance.

### **Figure 7.1. Cellular localisation of endogenous E2F-1**

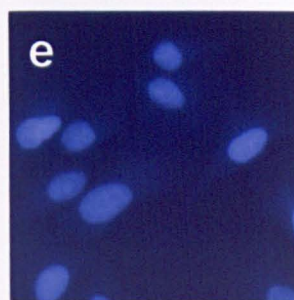
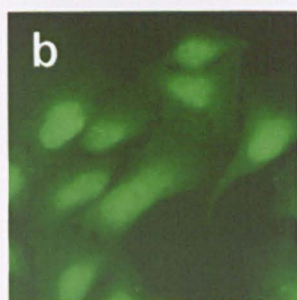
Representative fields from asynchronous MCF7 cells immunostained with the anti-E2F-1 antibody KH-95 and studied by indirect immunofluorescence (a, b and c). d, e and f show the same fields stained with DAPI. Cells were treated with etoposide (10 $\mu$ M) or UV-irradiation (50 J/m<sup>2</sup>) as indicated for 16 h prior to immunostaining. Images were captured at 40x magnification. For picture clarity the brightness of the images was increased.

$\alpha$  E2F-1

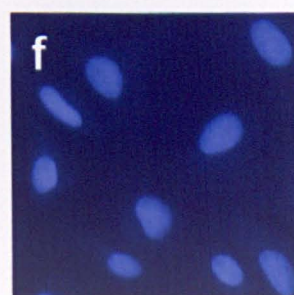
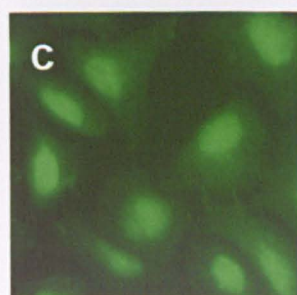
DAPI



-



+ UV



+ Et



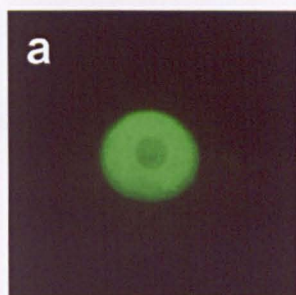
## **Figure 7.2. Cellular localisation of HA-E2F-1**

Representative fields from asynchronous MCF7 cells transfected with HA-tagged E2F-1 (5µg) and CMV-DP-1 (1µg), and studied by indirect immunofluorescence with the anti-HA antibody (HA-11; a, b and c). d, e and f show the same fields stained with DAPI. Cells were treated with etoposide (10µM) or UV-irradiation (50 J/m<sup>2</sup>) as indicated for 16 h prior to immunostaining. Images were captured at 60x magnification.

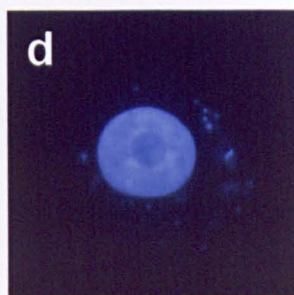
$\alpha$  HA

DAPI

a

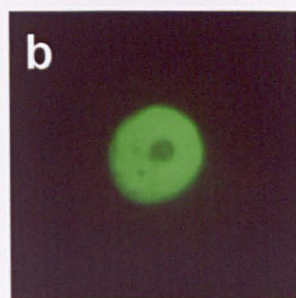


d

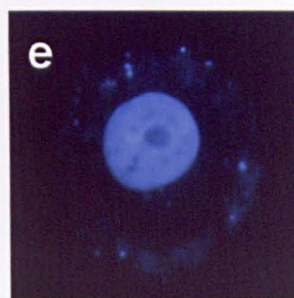


-

b

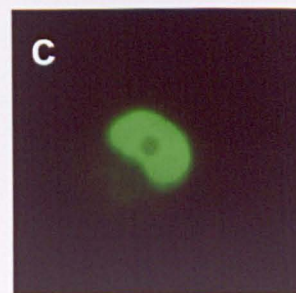


e

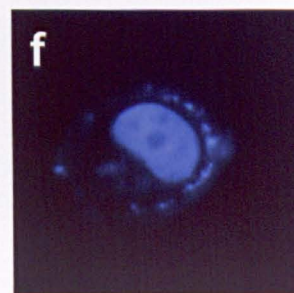


+ UV

c



f



+ Et

### **Figure 7.3. Cellular localisation of HA-S364A**

Representative fields from asynchronous MCF7 cells transfected with HA-tagged S364A (5µg) and CMV-DP-1 (1µg), and studied by indirect immunofluorescence with the anti-HA antibody (HA-11; a, b and c). d, e and f show the same fields stained with DAPI. Cells were treated with etoposide (10µM) or UV-irradiation (50 J/m<sup>2</sup>) as indicated for 16 h prior to immunostaining. Images were captured at 60x magnification.

Figure 5A Fluorescence microscopy

Fluorescence microscopy of cells expressing HA-tagged proteins

Cells were transfected with HA-tagged proteins and analyzed by fluorescence microscopy

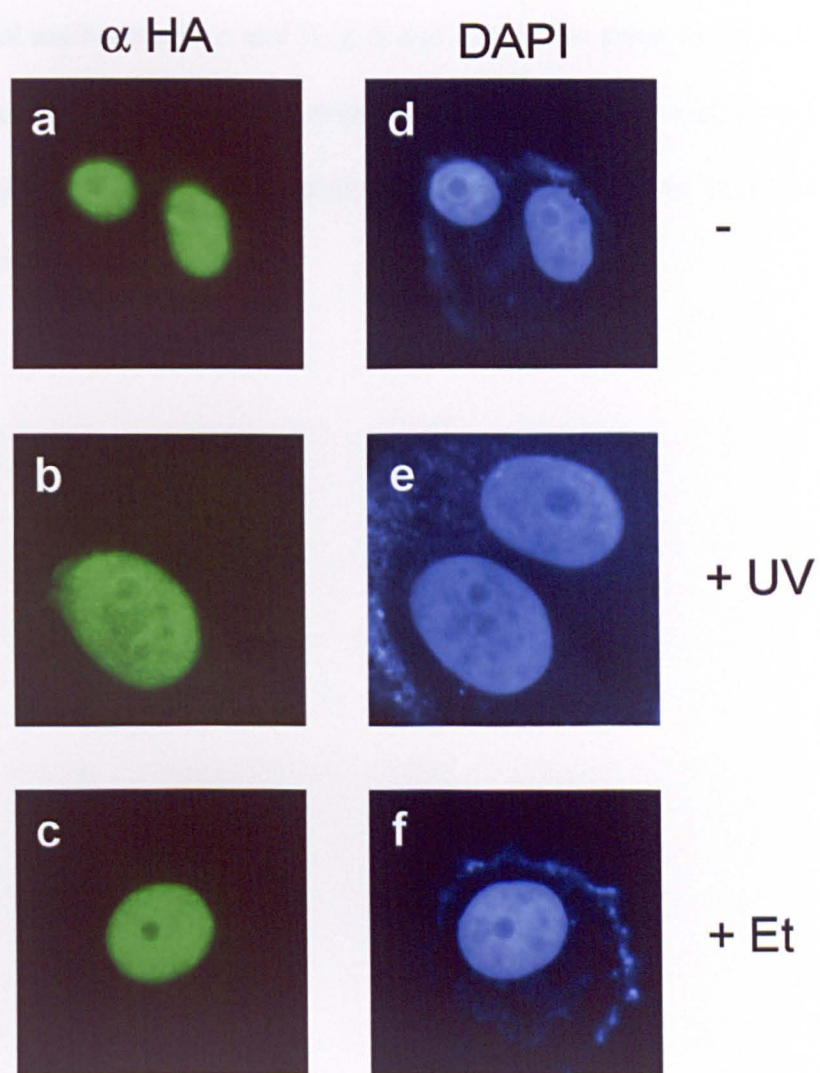
Cells were transfected with HA-tagged proteins and analyzed by fluorescence microscopy

Cells were transfected with HA-tagged proteins and analyzed by fluorescence microscopy

Cells were transfected with HA-tagged proteins and analyzed by fluorescence microscopy

Cells were transfected with HA-tagged proteins and analyzed by fluorescence microscopy

Cells were transfected with HA-tagged proteins and analyzed by fluorescence microscopy



#### **Figure 7.4. Co-expression of E2F-1 and Chk2 wild-type**

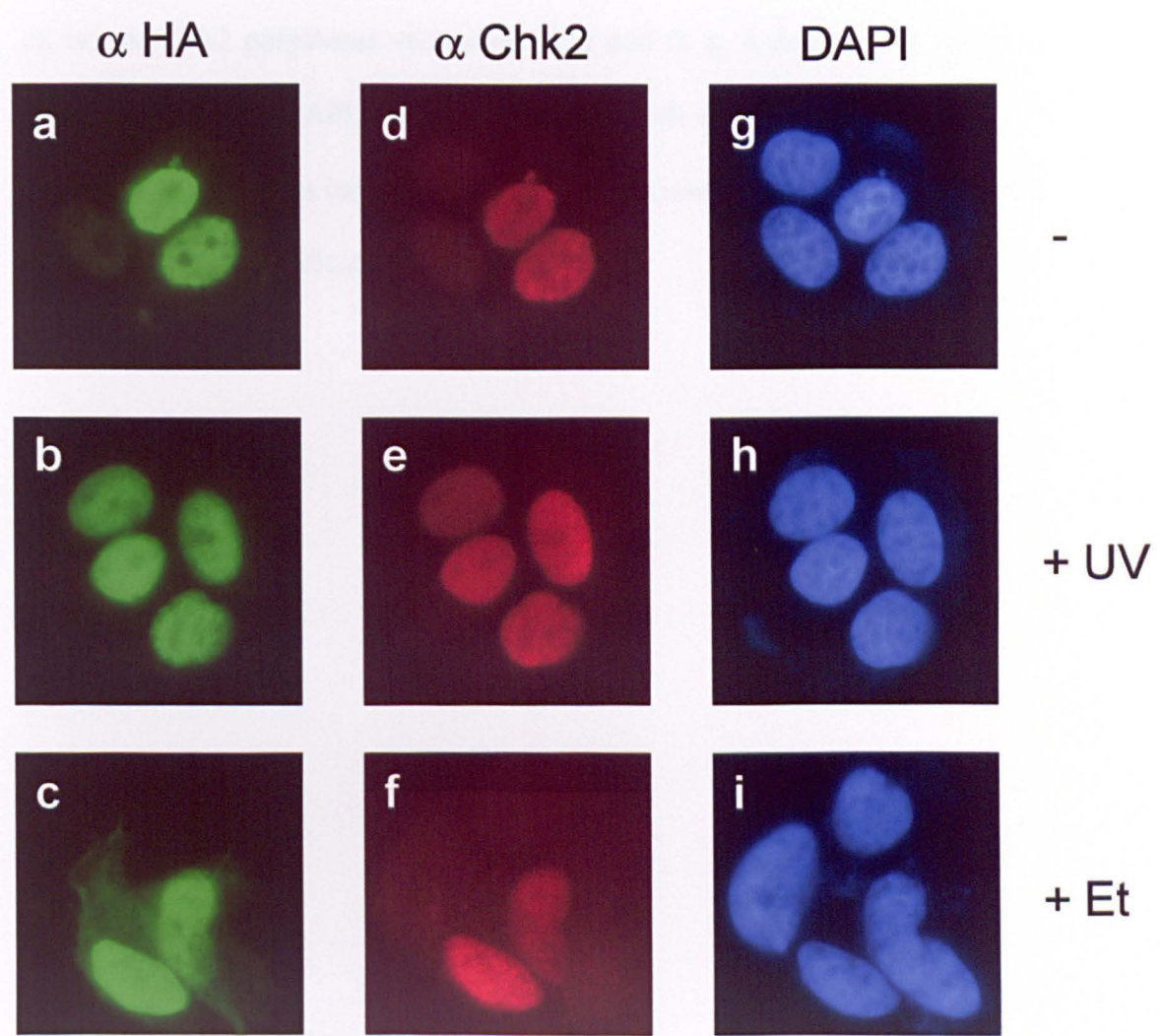
Representative fields from asynchronous U2OS cells transfected with HA-tagged E2F-1 (5 $\mu$ g) and CMV-DP-1 (1 $\mu$ g), and Flag-Chk2 wild-type (5 $\mu$ g), and immunostained with anti-HA monoclonal antibody (HA-11; a, b and c), or anti-Chk2 polyclonal antibodies (d, e and f). g, h and i show the same fields stained with DAPI. Cells were treated with etoposide (10 $\mu$ M) or UV-irradiation (50 J/m<sup>2</sup>) as indicated for 16 h prior to immunostaining. Images were captured at 60x magnification.

Figure 7.2. Localization of  $\alpha$ -HA and  $\alpha$ -Chk2 in cells.

Immunofluorescence images of cells expressing  $\alpha$ -HA and  $\alpha$ -Chk2.

Figure 7.2.1 shows the localization of  $\alpha$ -HA and  $\alpha$ -Chk2 in cells.

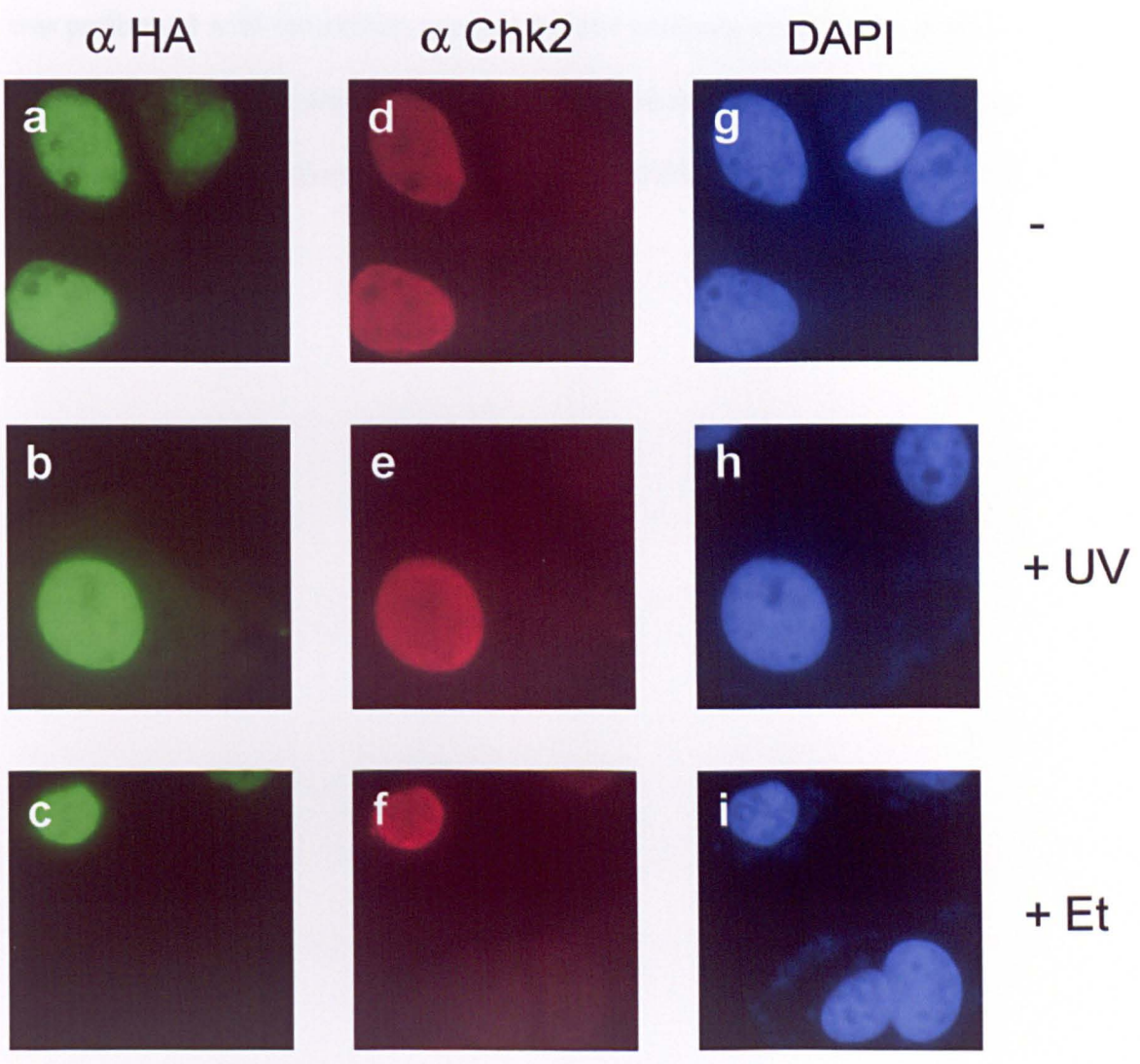
Figure 7.2.2 shows the localization of  $\alpha$ -HA and  $\alpha$ -Chk2 in cells.



### **Figure 7.5. Co-expression of E2F-1 and Chk2 dominant-negative**

Representative fields from asynchronous U2OS cells transfected with HA-tagged E2F-1 (5 $\mu$ g) and CMV-DP-1 (1 $\mu$ g), and Flag-Chk2 dominant-negative (5 $\mu$ g), and immunostained with anti-HA monoclonal antibody (HA-11; a, b and c), or anti-Chk2 polyclonal antibodies (d, e and f). g, h and i show the same fields stained with DAPI. Cells were treated with etoposide (10 $\mu$ M) or UV-irradiation (50 J/m<sup>2</sup>) as indicated for 16 h prior to immunostaining. Images were captured at 60x magnification.







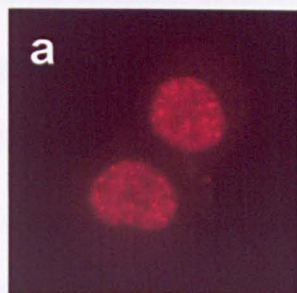
**Figure 7.6. Phosphorylated Ser364 E2F-1 in normal cells.**

Representative fields from asynchronous MCF7 cells immunostained with the affinity purified P-S364 antibody and studied by indirect immunofluorescence (a and b); e and f show the same fields stained with DAPI. Peptide competition was performed with the affinity purified P-S364 antibody either in the presence of the phosphorylated peptide (2 $\mu$ g) (c) or non-phosphorylated peptide (2 $\mu$ g) (d); g and h show the same fields stained with DAPI. Images were captured at 60x magnification.

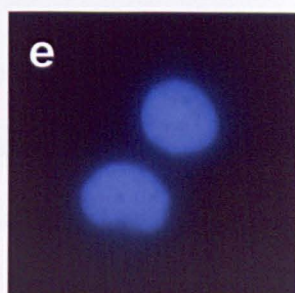
$\alpha$  P-S364

DAPI

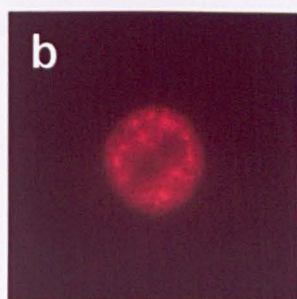
a



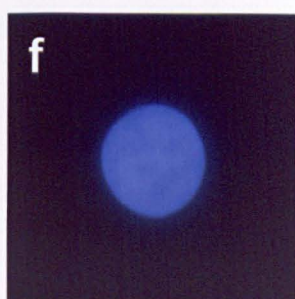
e



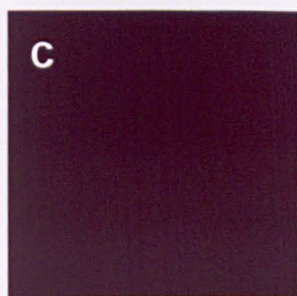
b



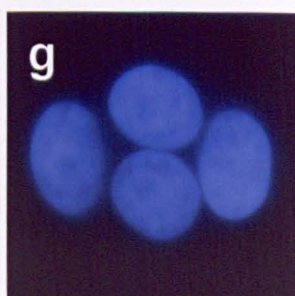
f



c

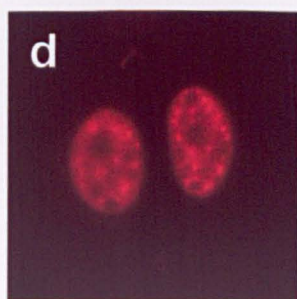


g

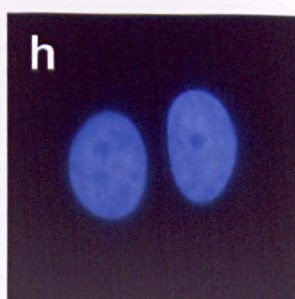


+ P-peptide

d



h



+ non  
P-peptide

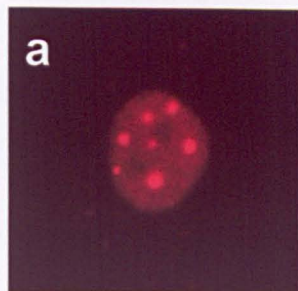
**Figure 7.7. Phosphorylated Ser364 E2F-1 in etoposide-treated cells.**

Representative fields from MCF7 cells treated with etoposide (10 $\mu$ M) for 16 h and immunostained with the affinity purified P-S364 antibody and studied by indirect immunofluorescence (a and b); e and f show the same fields stained with DAPI. Peptide competition was performed with the affinity purified P-S364 antibody either in the presence of the phosphorylated peptide (2 $\mu$ g) (c) or non-phosphorylated peptide (2 $\mu$ g) (d); g and h show the same fields stained with DAPI. Images were captured at 60x magnification.

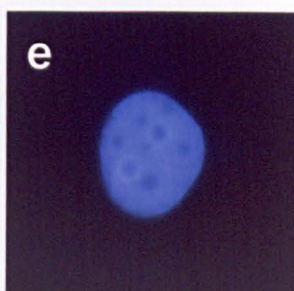
$\alpha$  P-S364

DAPI

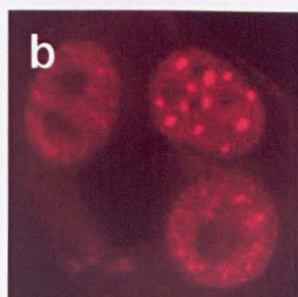
a



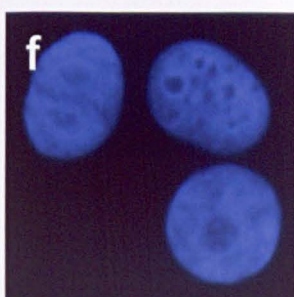
e



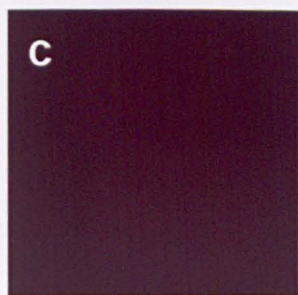
b



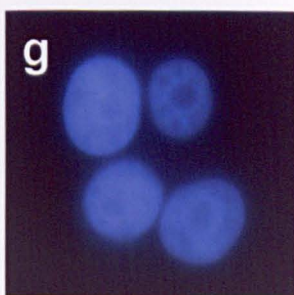
f



c

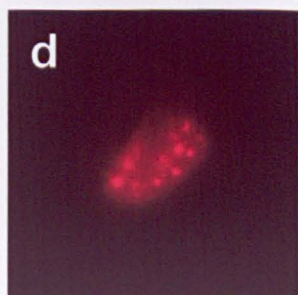


g

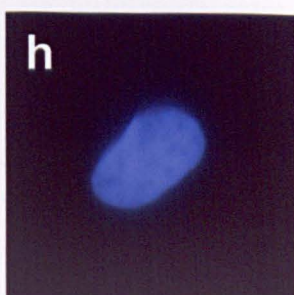


+ P-peptide

d



h



+ non  
P-peptide

### **Fig 7.8. Co-localisation of E2F-1 with PML**

Representative fields from asynchronous SAOS2 cells left untreated (a, e, i and m) or treated with etoposide (10 $\mu$ M) for 3 h (b, f, j and n); 8 h (c, g, k and o) or 16 h (d, h, l and p) and immunostained with affinity purified P-S364 antibody (a, b, c and d), or anti-PML monoclonal antibody (e, f, g and h). i, j, k and l show the merged P-S364 and PML fields. m, n, o, and p show the same fields stained with DAPI. Images were captured at 60x magnification.

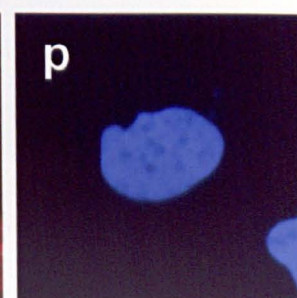
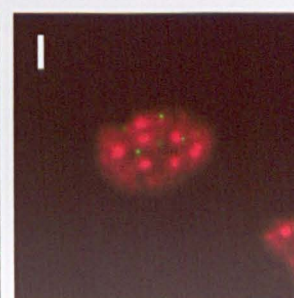
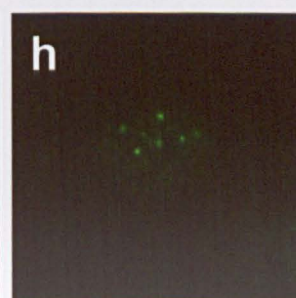
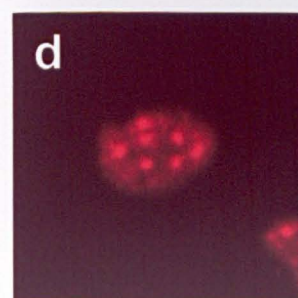
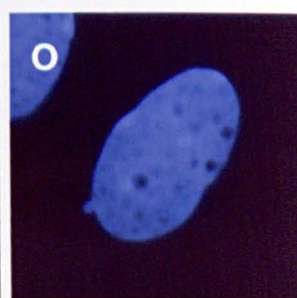
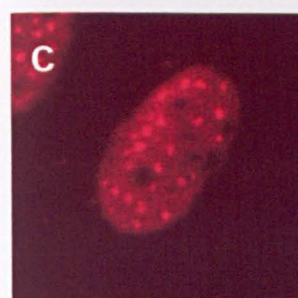
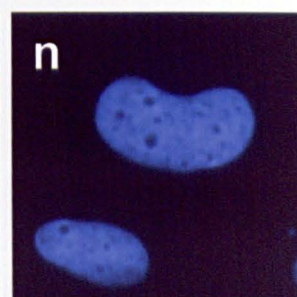
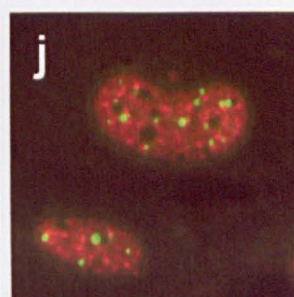
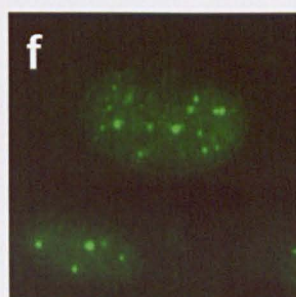
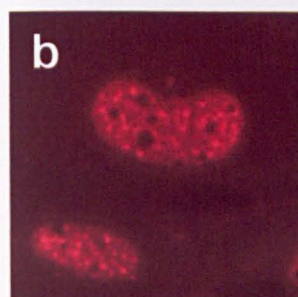
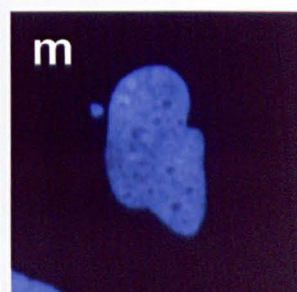
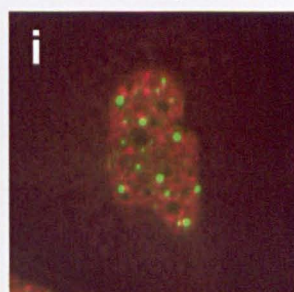
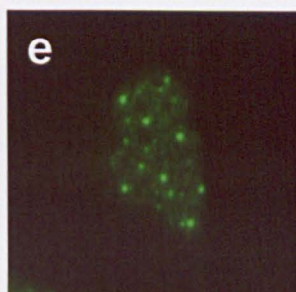
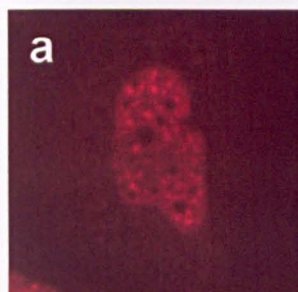


$\alpha$  P-S364

$\alpha$  PML

Merge

DAPI



# Chapter 8. Discussion

## 8.1. Introduction

It is well established that the E2F family of transcription factors plays a central role in co-ordinating cell cycle progression at the G1 to S-phase transition. In addition, the ability of E2F-1 to induce apoptosis provides the cell with a safeguard mechanism against oncogenic as well as genotoxic stress. The activity of E2F must be tightly regulated in order to ensure the timely activation and repression of E2F target genes during normal cellular growth. In response to DNA damage, cells must be able to arrest cell cycle progression and suppress the induction of apoptosis to allow time for DNA to be repaired. Conversely, if the damage is too severe cells must be able to efficiently activate apoptotic genes. In this study, phosphorylation of E2F-1, and the regulation of E2F-1 activity in response to DNA damage, has been investigated.

## 8.2. E2F-1 is regulated by the DNA damage signalling pathway

The proteins that detect and signal DNA damage have been the subjects of intense study over recent years. In the DNA damage signalling pathway, the related kinases ATM and ATR are regarded to be sensors of DNA damage, whereas the checkpoint kinases Chk1 and Chk2 function as effectors of the response (Durocher and Jackson, 2001). Despite the recent explosion of

information regarding the components of the cell cycle checkpoints, the mechanisms through which checkpoints arrest the cell cycle, repair DNA or induce apoptosis are not yet clear. Therefore, the identification of additional substrates of these kinases is particularly important.

Recent studies have shown that E2F-1 is regulated in response to DNA damage. For example, treating cells with DNA damaging agents such as those used in cancer chemotherapy, increases E2F-1 protein levels (Blattner *et al.*, 1999; O'Connor *et al.*, 2000; Lin *et al.*, 2001). Consistent with these reports, the results presented in chapter 3 show that E2F-1 is induced in response to various DNA damaging agents, in a variety of cell lines, and suggest that the induction of E2F-1 is a general response to DNA damage. The extent of induction did vary depending on the cell line and the drug being used suggesting that different signalling pathways were being activated. However, it is likely that a common mechanism is used by each of these pathways to regulate E2F-1 accumulation.

E2F-1 is specifically induced by DNA damage, as no change in the E2F-2 or E2F-3 family members was evident after treatment with etoposide or other DNA damaging agents (Figure 3.2, and data not shown). This strongly suggests that the induction of E2F-1 is not simply a consequence of enrichment of G1 arrested cells and constitutes a bone-fide part of the cells defence against DNA damage.

The observation that E2F-1 and p53 are up-regulated in response to the same DNA damaging agents with similar kinetics suggested that they may be commonly regulated by the DNA damage signalling pathway. Accumulation of p53 in response to IR requires both ATM and Chk2 (Siliciano *et al.*, 1997; Chehab *et al.*, 2000), therefore if E2F-1 and p53 are commonly regulated, then



it was expected that E2F-1 would also be subject to regulation by ATM and Chk2. During the course of this study the involvement of ATM in the phosphorylation and accumulation of E2F-1 in response to DNA damage was reported (Lin *et al.*, 2001). In agreement with this, E2F-1 failed to be induced by etoposide in a cell line deficient in ATM gene function. Consistent with the notion that ATM responds primarily to DNA d/s breaks (Abraham, 2001), E2F-1 protein rapidly accumulated in the same cells in response to replication inhibition induced by UV-irradiation.

Several lines of evidence are presented in support of the idea that Chk2 regulates E2F-1. First of all, a dominant-negative Chk2 blocked the induction of p53 and E2F-1 in response to etoposide treatment, with negligible effect on UV-irradiated cells. In HCT-15 cells, which are deficient in Chk2 gene function, E2F-1 failed to accumulate in response to treatment with etoposide, yet retained the ability to respond to UV-irradiation. Further evidence in support of this idea was gained by transfection of Chk2 into U2OS cells, which allowed a greater level of E2F-1 induction in response to etoposide treatment, and in HCT-15 cells, introduction of Chk2 restored the ability of E2F-1 to respond to etoposide. Taken together these studies confirm that the induction of E2F-1 in response to DNA damage involves ATM, and reveal a new role for Chk2 in the regulation of E2F-1 in response to DNA damage. More specifically, these results suggest that ATM and Chk2 are involved in signalling to E2F-1 in response to agents that induce DNA d/s breaks, and infer that E2F-1 is regulated in an ATM and Chk2 independent manner in response to replication inhibition caused by UV-irradiation.

At present, it is unclear how Chk2 and ATM co-operate to regulate E2F-1 activity, and more studies are needed to clarify the molecular mechanisms involved. However, it is interesting that other proteins involved in cell cycle checkpoint control, such as p53 and BRCA1, are similarly phosphorylated by both ATM and Chk2 (Canman *et al.*, 1998; Hirao *et al.*, 2000; Lee *et al.*, 2000; Cortez *et al.*, 1999). Moreover, several proteins that exist in the same complex can be phosphorylated by ATM and Chk2, for example p53 and MDM2 (Chehab *et al.*, 2000; Shieh *et al.*, 2000; Maya *et al.*, 2001), or BRCA1 and its inhibitory interacting partner CtIP (Lee *et al.*, 2000; Li *et al.*, 2000; Cortez *et al.*, 1999; Tibbets *et al.*, 2000).

Multiple phosphorylations may be required to recruit and activate effector proteins only when checkpoint responses are fully activated. For example, ATM phosphorylation may be required to prime a substrate for subsequent phosphorylation and activation by Chk2. Alternatively, the level of phosphorylation could be used to modulate protein function. For example, phosphorylation by ATM alone may be sufficient to cause cell cycle arrest, whereas induction of apoptosis requires both ATM and Chk2 phosphorylation. The fact that multiple phosphorylations apply mainly to targets with pleiotrophic cellular effects such as p53, BRCA1 and E2F-1 lends some weight to this idea.

The requirement for two interacting proteins to both be phosphorylated may provide a safeguard mechanism that prevents the spurious activation of checkpoints. In this respect it is interesting that MDM2, an E2F-1 interacting protein, is also phosphorylated by ATM and possibly Chk2 (Maya *et al.*, 2001; Shieh *et al.*, 2000). It will be interesting to see whether other E2F-1 interacting

proteins, such as DP-1 and pRb are also subject to phosphorylation control in response to DNA damage.

The observation that E2F-1 and p53 are both phosphorylated by ATM and Chk2 suggests that they may act in concert to induce cell cycle arrest or apoptosis. Given the role of E2F-1 in inducing p53 accumulation through activation of p19<sup>ARF</sup>, the effect of E2F-1 induction could be to amplify the accumulation of p53. E2F-1 could further contribute to the p53 apoptotic response through the activation of genes involved in apoptosis such as p73 and Apaf-1. Alternatively, when p53 is absent or non-functional through mutation, E2F-1 may compensate for p53 loss, thus providing the cell with a safeguard mechanism. Consistent with this idea, cells that lack p53 can undergo cell cycle arrest (Agami and Bernards, 2000) and E2F-1 can induce apoptosis independently of p53 through the transcriptional activation of genes involved in apoptosis such as p73 and Apaf-1.

Functional redundancy exists within the DNA damage signalling pathway (Bartek and Lukas, 2003), and the possibility that E2F-1 may be subject to regulation by ATR and Chk1 can not be excluded. Accumulation of p53 in response to UV-irradiation requires functional ATR (Tibbets *et al.*, 1999), and Chk1 can phosphorylate p53 on Ser20 *in vitro* (Shieh *et al.*, 2000). Given the similarities in the regulation of p53 and E2F-1, it is likely that E2F-1 is also subject to regulation by ATR and Chk1 in response to UV-irradiation. In support of this idea, E2F-1 can be phosphorylated *in vitro* by ATR on Ser31 (Lin *et al.*, 2001). The fact that gene disruption of ATR or Chk1 causes embryonic lethality in the mouse has hampered studies into the role of ATR and Chk1 in checkpoint signalling. More studies are needed to address the role of

these proteins in signalling to E2F-1, perhaps by using siRNA to knock down gene function in human cells.

To conclude, the results presented in chapter 3 suggests that E2F-1 and p53 are commonly regulated by ATM and Chk2 in response to agents that cause DNA d/s breaks, and establish E2F-1 as a likely effector protein in the cellular response to DNA damage.

### **8.3. E2F-1 is phosphorylated on Ser364 by Chk2**

To further explore the mechanism for the involvement of Chk2 in the accumulation of E2F-1, the possibility that E2F-1 might be a direct substrate of Chk2 was investigated. Several lines of evidence are presented in chapter 4 suggesting that Chk2 phosphorylates E2F-1. The Chk kinases are Ser/Thr specific kinases for which a small number of physiological substrates have been identified (Bartek and Lukas, 2003). The consensus amino acid sequence for phosphorylation by Chk kinases has been determined (Hutchins *et al.*, 2000; O'Neill *et al.*, 2002; Seo *et al.*, 2003), and this was used to search the amino-acid sequence of E2F-1. Serine residue 364 in the C-terminal half of the protein was identified as fitting the consensus Chk phosphorylation site. The amino acids surrounding Ser364 are more similar to identified Chk2 kinase substrates such as Cdc25A, Cdc25C, BRCA1 and PML, than to p53. It has been suggested recently that two different classes of Chk2 substrate may exist, those similar to Cdc25 and others more similar to p53 (Bartek and Lukas, 2003). Whilst Cdc25C serves as a good substrate for Chk2 *in vitro*, activation of Chk2 as a

kinase towards p53 requires allosteric changes in Chk2 induced by its binding to full-length p53 (Craig *et al.*, 2003).

To investigate whether E2F-1 is regulated by Chk2 phosphorylation, a mutational analysis was performed at the potential Chk2 phosphorylation site. Phosphorylation of GST-E2F-1 *in vitro* with purified His-Chk2 results in a mobility shift of E2F-1 in SDS-PAGE that is not apparent with the S364A mutant derivative. However, an equivalent mobility shift of E2F-1 and S364A was observed when cyclinA/cdk2 purified from baculovirus-infected *Sy9* cells was used as the source of kinase. These results infer that the absence of the mobility shift observed with S364A when incubated with Chk2 results from altering a specific Chk2 kinase site.

Phosphorylation of GST-E2F-1 *in vitro* with purified His-Chk2 results in the labelling of E2F-1 with  $\gamma$   $^{32}\text{P}$  ATP to a level comparable with Cdc25C, in contrast to the S364A mutant that failed to be phosphorylated. The observation that E2F-1 and S364A could be phosphorylated to about equivalent efficiency by cyclinA/cdk2 consolidated the previous results suggesting that the absence of S364A phosphorylation results from altering a specific Chk2 kinase site.

While E2F-1 does not fall into the same class of substrate as p53, it can form a complex with Chk2 (see chapter 5). Thus it is possible that the activation of Chk2 as a kinase towards E2F-1 is dependent on its ability to bind to full length E2F-1. It will be interesting to compare phosphorylation of the full-length E2F-1 protein with the C-terminal part of E2F-1, or an E2F-1 peptide that contains the Ser364 site. Furthermore, the fact that E2F-1 and Chk2 can form a complex in cells may explain why E2F-1 fails to become stabilised in HCT-15 cells.

To establish whether Chk2 phosphorylates Ser364 of E2F-1 *in vivo*, an anti-phospho-specific antiserum directed against the consensus site in which Ser364 resides was prepared. First the reactivity of the antibody was tested on *in vitro* phosphorylated E2F-1. The antibody reacted strongly with E2F-1 phosphorylated *in vitro* by Chk2 compared to un-phosphorylated E2F-1, and had a greatly reduced activity towards the S364A derivative. Inclusion of the phosphorylated peptide, and not the unphosphorylated peptide efficiently competed out the signal confirming the reactivity of the antibody for phosphorylated Ser364 was specific. These results support the view that the Ser364 site is phosphorylated by Chk2 kinase, and importantly that the P-S364 antibody recognises the phosphorylated site in E2F-1.

Next, the anti-P-S364 antibody was used to establish that Ser364 is subject to phosphorylation control *in vivo* in response to DNA damage caused by etoposide treatment. Because E2F-1 is stabilised in response to etoposide treatment, it was possible that any increase in reactivity observed with the P-S364 antibody would simply reflect an increased abundance of E2F-1 protein. To exclude this possibility, cells were immunoblotted with P-S364 under experimental conditions in which there was little change in the steady state level of E2F-1. Under these experimental conditions the antiserum clearly reacted with E2F-1 in etoposide treated cells, but not in non-stressed cells.

Although the polypeptide recognised by P-S364 migrated to exactly the same size as the polypeptide recognised by the general E2F-1 antibody (KH-95), it cannot be taken as proof that they are the same. Therefore, to confirm that the antibody is recognising phosphorylated E2F-1, immunoprecipitation was performed with the general E2F-1 antibody KH-95 prior to western blot

analysis with P-S364. Immunoprecipitated E2F-1 was clearly recognised by P-S364 only in etoposide treated cells. Taken together, these findings suggest that Chk2 phosphorylates E2F-1 on Ser364 *in vitro* and on the same site *in vivo* in response to DNA damage, and firmly establish E2F-1 as a physiological target for Chk2 kinase.

A comparison of amino-acid sequences of the different E2F family members reveals that the serine at 364 is unique to E2F-1. This suggests that E2F-1 is the only E2F family member that is phosphorylated by Chk2, although it remains possible that Chk2 may phosphorylate different sites on the other E2F family members. In addition, Chk2 shares overlapping substrate specificity *in vitro* with the other identified checkpoint kinase Chk1, thus there is a distinct possibility that the same site on E2F-1 will be phosphorylated by Chk1 *in vivo* in response to a different kind of DNA damaging agent, such as UV-irradiation.

#### **8.4. Phosphorylation of Ser364 regulates E2F-1 stability**

The mechanisms that control the accumulation of E2F-1 activity in normally growing cells are complex and involve transcriptional control of the E2F-1 promoter (Hsiao *et al.*, 1994; Johnson *et al.*, 1994; Sellers *et al.*, 1995), control of E2F-1 DNA-binding activity through the action of cyclinA/cdk2 (Krek *et al.*, 1994), and control of E2F-1 protein stability through ubiquitin-mediated degradation by the proteasome (Hateboer *et al.*, 1996).

In chapter 5 the mechanism through which E2F-1 is induced was investigated. First, the S364A mutant devoid of Chk2 phosphorylation was compared with E2F-1 in its ability to respond to DNA damage. The comparison

of exogenous E2F proteins revealed that the temporal accumulation of S364A was about 3-4 times less than E2F-1 under similar conditions of etoposide treatment, indicating that Ser364 plays a significant role in the regulation of E2F-1 stability. The S364A mutant does still retain some ability to respond to DNA damage, and it is likely that this is through the ATM phosphorylation site. A double mutant containing S364A and S31A should therefore be unresponsive to agents that cause DNA d/s breaks, and will be a useful mutant to have for future studies aimed at unravelling the contribution of ATM and Chk2 to E2F-1 induction.

A possible explanation for the increase in E2F-1, but not S364A abundance in response to etoposide treatment is that Chk2 controls the stability of E2F-1. Although the stability of E2F-1 and S364A were similar in the absence of etoposide, S364A was significantly less stable in the presence of etoposide. The observation that E2F-1 induction in response to DNA damage reflects an increase in protein stability suggests that the mechanism responsible for the normal turnover of E2F-1 in cells is being disrupted.

Two activities have been implicated in the control of E2F-1 degradation, the binding of pRb to the carboxy terminus of E2F-1 (Hateboer *et al.*, 1996; Hofman *et al.*, 1996), and the binding of p45<sup>SKP2</sup> to the amino-terminal sequences of the protein (Marti *et al.*, 1999). In response to DNA damage and other stress signals, pRb becomes hypo-phosphorylated (Ameltem *et al.*, 1996; Krtolica *et al.*, 1998). The increase in p53 in response to stress signals can cause the hypo-phosphorylation of pRb via the induction of the cdi p21, which can inhibit the phosphorylation of pRb via cdks. As E2F-1 favours the binding to hypo-phosphorylated pRb, in response to DNA damage, it was anticipated that



there would be an increase in the amount of E2F-1 in complex with pRb. An increase in the pRb/E2F-1 complex could be responsible for the accumulation of E2F-1 in response to DNA damage, since pRb binding can protect E2F-1 from ubiquitination (Hofman *et al.*, 1996).

Several lines of evidence are presented to suggest that pRb is not responsible for the induction of E2F-1. Results presented in chapter 3 showed that E2F-1 is still induced in SAOS2 cells which lack both p53 and pRb proteins, and argue strongly that the accumulation of E2F-1 in response to DNA damage can be independent of p53 and pRb. Moreover, E2F-1 and S364A form a complex with pRb *in vitro* and *in vivo* implying that phosphorylation by Chk2 stabilises E2F-1 in a pRb-independent manner.

Consistent with pRb becoming hypo-phosphorylated in response to DNA damage (Ameltem *et al.*, 1996; Krtolica *et al.*, 1998), an increase in the pRb/E2F-1 complex is observed in response to etoposide treatment. As pRb binds to a region within the E2F-1 transactivation domain, this infers that the induced E2F-1 is held in a transcriptionally inactive state. However, the ratio of E2F-1 complexed with pRb is similar in stressed and non-stressed cells. Since E2F-1 is still transcriptionally active in non-stressed cells, this result suggests that a population of E2F exists in stressed cells that is not complexed with pRb, and thus free to activate gene transcription.

Recently, p45<sup>SKP2</sup> was reported to bind to the amino-terminus of E2F-1 and target E2F-1 for degradation (Marti *et al.*, 1999). It has been suggested recently that ATM-mediated phosphorylation might alter the degradation of E2F-1 by inhibiting the binding and function of Skp2 and thus prevents the normal degradation of E2F-1, however this is only speculative and no

experimental evidence exists to support this idea (Lin *et al.*, 2001). Nonetheless, p45<sup>SKP2</sup> remains an attractive candidate, and future studies should address the contribution that ATM and Chk2 make to the E2F-1/Skp2 interaction.

Interestingly, it has also been shown that E2F-1 binds and co-precipitates with MDM2, and a domain in E2F-1 shows striking similarity to the MDM2 binding domain of p53 (Martin *et al.*, 1995). The interaction of MDM2 with p53 through this domain is required for the degradation of p53. In response to DNA damage, phosphorylation on Thr18 and Ser20 of p53 by Chk2 disrupts its interaction with MDM2 (Craig *et al.*, 1999; Schon *et al.*, 2002; Chehab *et al.*, 2000). In addition, phosphorylation of MDM2 by ATM has been suggested to disrupt its interaction with p53 leading to p53 accumulation (Maya *et al.*, 2001). Given that E2F-1 and p53 are similarly regulated by ATM and Chk2 (chapter 3), it is possible that disruption of the MDM2/E2F-1 interaction mediated by Chk2 phosphorylation plays a major role in the induction of E2F-1 in response to genotoxic stress. In support of this idea, Ser364, the site phosphorylated by Chk2 is next to the MDM2 binding domain, and MDM2 can act as an inhibitor of E2F-1 induced apoptosis (Mason *et al.*, 2001) and can target both E2F-1 and DP-1 proteins for degradation (Loughran and La Thangue, 2000).

It was recently shown that polyubiquitination of p53, which is required for degradation by the ubiquitin proteasome pathway, requires the activities of both p300 and MDM2 (Grossman *et al.*, 2003). E2F-1 also interacts with and is acetylated by p300 (Trouche *et al.*, 1996; Martinez-Balbas *et al.*, 2000). The consequence of E2F-1 acetylation was to potentiate E2F-1 activity by increasing its DNA-binding ability, *transactivation* potential, and extension of its protein half-life. Although extension of E2F-1 half-life may seem counter-intuitive to

p300 being involved in the ubiquitination and degradation of E2F-1, it is worth noting that similarly to E2F-1, acetylation of p53 by p300 has been reported to potentiate p53 DNA-binding and transcriptional activity (Gu and Roeder, 1997; Dumaz and Meek, 1999). Thus it remains a distinct possibility that p300 plays a role, possibly in combination with MDM2 in the regulation of E2F-1 stability.

P19<sup>ARF</sup> has also been implicated in the regulation of E2F stability, and has been shown to target certain E2F species including E2F-1 for degradation. P19<sup>ARF</sup> induced degradation of E2F-1 depends on a functional proteasome, therefore ARF mediated E2F degradation may be connected with E2F ubiquitination (Hofman *et al.*, 1996; Hateboer *et al.*, 1996; Campanero and Flemming, 1997; Marti *et al.*, 2001), where ARF might act as an adapter that brings E2F-1 and the ubiquitination apparatus together. ARF can localise to nucleoli along with MDM2, and whether MDM2 is the ubiquitin ligase involved in the degradation of E2F-1 by ARF remains to be determined, although a necessary role for MDM2 in E2F-1 degradation has been suggested (Blattner *et al.*, 1999).

Thus, ARF, MDM2 and p300 are proteins that associate with E2F-1 and can regulate its function, and may be involved in the regulation of its stability. The availability of cells deficient in MDM2, ARF or p300 gene function would greatly assist in determining the contribution made by these proteins to the induction of E2F-1 in response to DNA damage.

The observation that Chk2 and E2F-1 form a complex *in vitro* and under physiological conditions infers that in addition to phosphorylation, Chk2 may regulate E2F-1 through additional mechanisms. In analogy to pRb, Chk2 may stably bind to E2F-1 and mask a signal for degradation, or the formation of a

transient complex may induce a conformational change that is sufficient to disrupt E2F-1s interaction with other proteins such as MDM2.

Interestingly, it has been shown recently that phosphorylation of E2F-1 at residues 403 and 433 can down-regulate its stability (Vandel and Kouzarides, 1999). Therefore, stress signals may increase the stability of E2F-1 by inhibiting the phosphorylation of E2F-1 at these sites. This raises the possibility that E2F-1 stability may also be regulated by protein phosphatases that become activated in response to DNA damage.

In summary, although evidence has been presented that links Chk2 to the regulation of E2F-1 stability in response to DNA damage, more studies are required to determine the mechanism. However it is likely that it will involve the modulation of E2F-1s interaction with another cellular protein that remains to be determined.

## **8.5. Regulation of E2F-1 activity by Chk2**

Phosphorylation can affect the activity of a transcription factor by modulating its DNA-binding activity and its interaction with co-activators and components of the basal transcription machinery (Hill and Triesman, 1995). Gel retardation showed that the mutant derivative of E2F-1 that is incapable of phosphorylation on Ser364 was competent in its ability to form a heterodimer with DP-1 and bind to the E2F-recognition site. Moreover, E2F-1 and S364A DNA-binding were unaffected by etoposide treatment. Taken together, these results imply that phosphorylation at Ser364 does not regulate E2F-1 DNA-binding activity. However, more studies using the P-S364 antibody in gel

retardation or Chip assays are required to properly address this question. Importantly, these results do demonstrate that the integrity of E2F-1 was not altered by mutagenesis.

The results presented in chapter 6 demonstrate that Ser364 is important for the transcriptional activity of E2F-1. S364A has a reduced transcriptional activity when assessed for its ability to activate several E2F-responsive promoters under non-stress conditions. Furthermore, the rise in reporter activity in response to increasing amounts of S364A was not as significant as that detected when equivalent amounts of E2F-1 were transfected. There are a number of possible explanations for this observation. One possibility is that S364A has a lower affinity for an endogenous protein involved in transcriptional activation, such as p300/CBP or Sp1 (Trouche *et al.*, 1996; Morris *et al.*, 2000; Dyson, 1998), and thus can not efficiently recruit it to the promoter. Another possibility is that S364A can not efficiently interact with components of the basal transcriptional machinery, such as TBP (Dyson, 1998; Ross *et al.*, 1999). Alternatively, transfection may stress the cells enough for Chk2 to become activated, or low levels of active Chk2 may exist under non-stress conditions (DiTullio *et al.*, 2002). Consistent with this idea, low levels of Ser364 phosphorylated E2F-1 are detected by immunofluorescence in non-stressed cells (Figure 7.6). Having ruled out other possible explanations, such as differences in protein expression, DNA-binding or rate of degradation, the idea that phosphorylation of E2F-1 on Ser364 plays a role in recruiting co-activator proteins to the promoter, or promotes the assembly of the transcription initiation complex warrants further investigation.

Under normal conditions, the steady state levels of E2F-1 and S364A are the same. However, etoposide treatment significantly increased the stability and half-life of E2F-1, but not the S364A mutant (see chapter 5). Therefore, under DNA damage conditions, it was expected that the rise in E2F-1 protein levels would be accompanied by an increase in the level of transcription. Contrary to expectation, etoposide treatment failed to stimulate E2F-1-dependent gene expression from the ARF promoter. Conversely, there was a decrease in both E2F-1 and S364A dependent transcription. This was not due to a general shut down of transcription as the expression of the internal control,  $\beta$ -gal, was unaffected. Therefore hypo-phosphorylation of pRb and the subsequent increase in binding to E2F proteins could account for the repression of E2F-dependent transcription observed.

If phosphorylation of Ser364 augments E2F-1-dependent transcription, then under conditions of etoposide treatment in which exogenous Chk2 is activated, the co-expression of Chk2 or Chk2 dominant-negative was expected to regulate E2F-1 dependent transcription. Unexpectedly, co-transfection of Chk2 or Chk2 dominant-negative had no effect on E2F-1 dependent transcription.

It is difficult to draw a conclusion from these experiments, partly due to the lack of a suitable positive control. However the use of an integrated reporter gene, and a cell line deficient in pRb may be necessary when looking at the effect that DNA damage has on E2F-1 dependent transcription.

Having established E2F-1 as a substrate of Chk2 (chapter 4), and that phosphorylation of Ser364 by Chk2 regulates E2F-1 stabilisation (chapter 5), the consequence of increased E2F-1 abundance observed in response to DNA

damage was connected with apoptosis. In SAOS2 tumour cells, which carry an inactive p53 gene, over-expression of E2F-1 increased apoptosis after etoposide treatment significantly more than the S364A mutant lacking the Chk2 phosphorylation site. Moreover, the expression of the dominant-negative Chk2 mutant, Asp347Ala, reduced the level of apoptosis induced by E2F-1, but had no effect on S364A, thus providing evidence that E2F-1-dependent apoptosis is regulated by Chk2 phosphorylation.

Additionally, in SAOS2 cells, induction of E2F-1 correlated well with E2F-1 target genes involved in apoptosis such as Apaf-1, p73 and ARF. These results argue that induced E2F-1 protein is transcriptionally active, and support the idea that a population of E2F-1 exists that is not complexed with hypo-phosphorylated pRb. Therefore, the compromised ability of S364A to induce apoptosis likely reflects its inability to properly activate the transcription of these genes. These results provide a plausible pathway through which Chk2 can directly act to increase E2F-1 activity and stimulate the transcription of E2F target genes involved in apoptosis.

## **8.6. Mechanism of E2F-1 induced apoptosis**

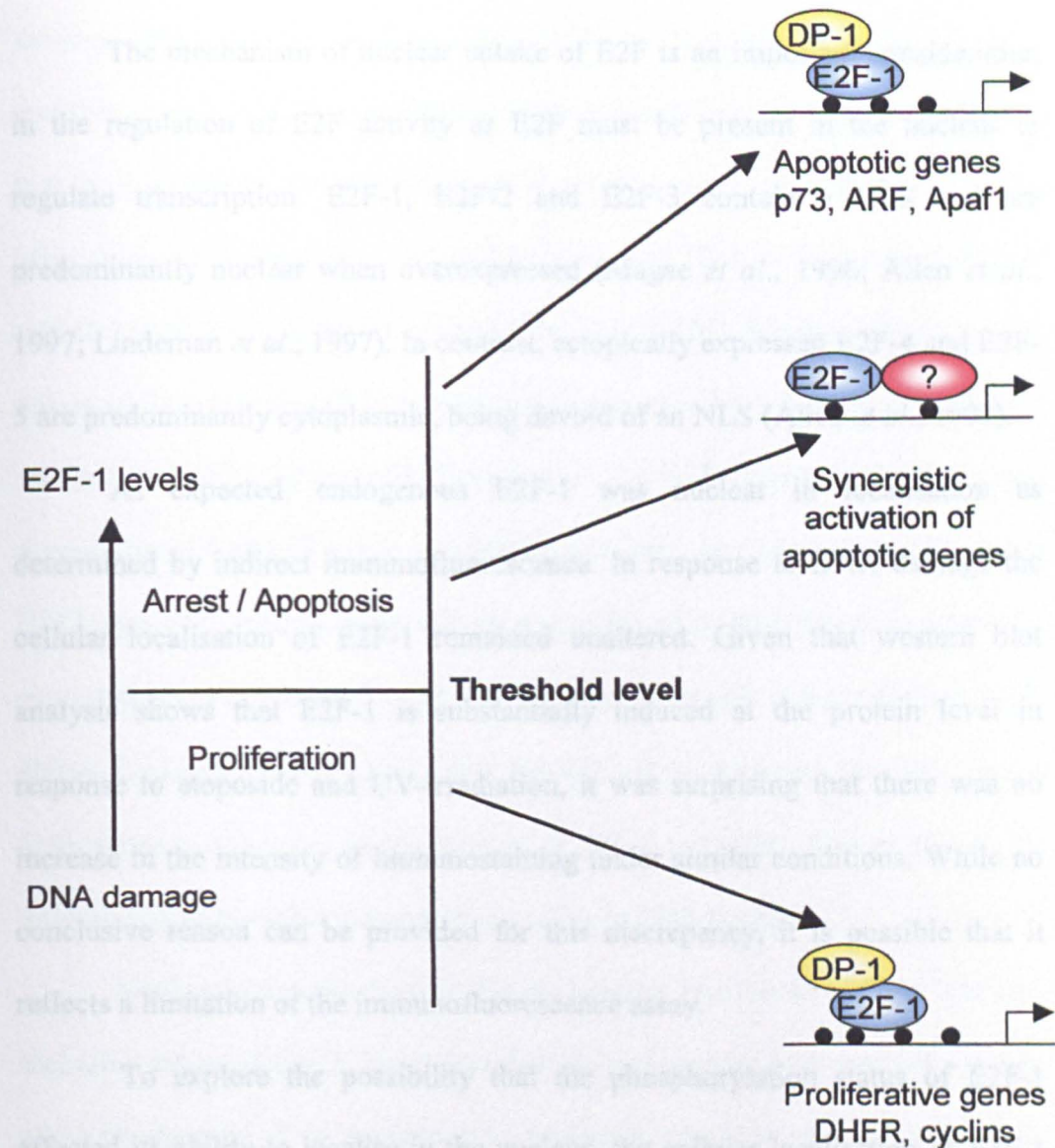
Whilst Chk2 phosphorylation provides a signal for E2F-1 to induce apoptosis, the mechanism through which E2F-1 causes apoptosis rather than cell cycle progression remains unclear. Nevertheless, it is believed that different target promoters may be occupied by distinct E2F sub-units, for example as cells progress through the cycle, but to date there is little evidence to support any promoter-specificity of complexes (Takahashi *et al.*, 2000). Against this

background, it remains possible that the level of E2F-1 protein influences its outcome in cells, namely proliferation or apoptosis, through a transcription-based mechanism. For example, a threshold level, such as the high level reached in response to DNA damage, could determine whether apoptosis occurs. Mechanistically, under these conditions a different, even overlapping, spectrum of genes may be activated compared to those involved in cell cycle progression, perhaps through binding to promoters containing lower affinity or diverged sequences within the binding sites (Figure 8.1). Alternatively, DNA damage may promote the interaction of E2F-1 with other cellular proteins to synergistically activate transcription of apoptotic target genes (Figure 8.1). In support of this idea, the YY1-binding protein RYBP was identified as a factor that binds to E2F-2 and E2F-3 and recruits these E2Fs to a subset of E2F target promoters containing YY1 binding sites (Schlisio *et al.*, 2002). Additionally, the physical interaction of the E-box factor TFE3 and E2F-3 facilitates transcriptional activation of the p68 gene which has both E2F binding sites and E-box elements within its promoter (Giangrande *et al.*, 2003). Damage-dependent acetylation of E2F-1 has been suggested to target E2F-1 to the p73 promoter (Pediconi *et al.*, 2003), and it will be interesting to understand how the different DNA damage-dependent post-translational modifications of E2F-1 are integrated. An important goal for future studies will be to use Chip assays to probe target gene specificity under DNA damage conditions. In this respect, use of the P-S364 antibody should yield some fascinating results.



## 8.7. Cellular localisation of E2F-1

Figure 8.1



**Figure 8.1 Mechanism of E2F-1 induced apoptosis**

Under normal circumstances, E2F-1 regulates the expression of genes required for cell cycle progression. In response to DNA damage, E2F-1 is stabilised and reaches a threshold level allowing the binding and activation of a different spectrum of genes, such as those involved in the induction of apoptosis. This could be achieved by the binding of E2F-1/DP complexes to lower affinity promoters, perhaps with diverged sequences in their binding sites. Alternatively, DNA damage may promote the interaction of E2F-1 with other cellular proteins to synergistically activate transcription of apoptotic genes.

## 8.7. Cellular localisation of E2F-1

The mechanism of nuclear uptake of E2F is an important consideration in the regulation of E2F activity as E2F must be present in the nucleus to regulate transcription. E2F-1, E2F-2 and E2F-3 contain a NLS and are predominantly nuclear when overexpressed (Magae *et al.*, 1996; Allen *et al.*, 1997; Lindeman *et al.*, 1997). In contrast, ectopically expressed E2F-4 and E2F-5 are predominantly cytoplasmic, being devoid of an NLS (Allen *et al.*, 1997).

As expected, endogenous E2F-1 was nuclear in localisation as determined by indirect immunofluorescence. In response to DNA damage the cellular localisation of E2F-1 remained unaltered. Given that western blot analysis shows that E2F-1 is substantially induced at the protein level in response to etoposide and UV-irradiation, it was surprising that there was no increase in the intensity of immunostaining under similar conditions. While no conclusive reason can be provided for this discrepancy, it is possible that it reflects a limitation of the immunofluorescence assay.

To explore the possibility that the phosphorylation status of E2F-1 affected its ability to localise in the nucleus, the cellular localisation of E2F-1 and the S364A mutant derivative was determined. The cellular localisation of the S364A mutant derivative did not differ from E2F-1 in either the absence or presence of etoposide or UV-irradiation. Moreover, co-transfection of Chk2 or the dominant negative-derivative of Chk2 did not affect the localisation of E2F-1 or S364A under any of the conditions tested. Therefore, phosphorylation of E2F-1 on Ser364 does not effect the ability of E2F-1 to localise to the nucleus.

Interestingly, use of the P-S364 antibody revealed that E2F-1, when phosphorylated by Chk2, localised to distinct nuclear bodies with intense staining. Given the size and frequency of the nuclear bodies, it was reasoned that they may be PML nuclear bodies. PML nuclear bodies are nuclear domains that are specifically disrupted in human acute promyelocytic leukemia (APL) cells. A typical mammalian nucleus has ~10 to 20 PML bodies, which vary in size (Lamond and Earnshaw, 1998). The role of PML bodies remains largely unknown, however several components of the nuclear body have been identified and include CBP, pRb and p53 (Zhong *et al.*, 2000). Furthermore, PML is itself a substrate of Chk2 (Yang *et al.*, 2002), and PML has been shown to recruit Chk2 into PML bodies, where it enhances the p53-Chk2 interaction thereby neutralising the inhibitory effects that MDM2 has on p53 (Louria-Hayon *et al.*, 2003). Co-localisation studies using P-S364 and an antibody directed against PML failed to show any co-localisation of E2F-1 with PML thus ruling out the possibility that phosphorylated E2F-1 resides in PML bodies.

Many proteins involved in the recognition and repair of DNA damage, including activated Chk2, localise to distinct nuclear foci most probably at sites of DNA damage. It is attractive to speculate therefore that in addition to regulating apoptosis, E2F-1 may play a role in the detection of DNA damage, and its subsequent repair. In support of this idea, the Mre11-Rad50-Nbs1 checkpoint protein complex that is involved in responses to DNA d/s breaks (D'Amours and Jackson, 2002) associates with E2F-1 through the Nbs-1 N-terminus (Maser *et al.*, 2001). Furthermore, the Nbs1/E2F-1 interaction occurs near origins of replication, suggesting that E2F is required to target the Mre11 complex to origins of replication to suppress origin firing in the presence of

damaged DNA. Moreover, *Drosophila* E2F and DP are present in origin recognition complexes (Bosco *et al.*, 2001), implying a direct role for E2F-1 in the regulation of DNA replication.

A recent study demonstrated that TopBP1 relocalises E2F-1 to nuclear foci along with BRCA1 in response to DNA damage. Upon DNA damage TopBP1 is recruited to stalled replication forks, where it participates in a DNA damage checkpoint. The recruitment of E2F-1 to foci along with the BRCA1-repair complex and TopBP1 further supports the idea that E2F-1 is directly involved in DNA damage checkpoint/repair and replication control in mammalian cells (Liu *et al.*, 2003). Interestingly the E2F-1 immunostaining pattern observed by Liu and colleagues is very similar to that of P-S364 E2F-1, therefore future studies aimed towards looking at the interaction of phosphorylated S364-E2F-1 with TopBP1 and BRCA1 should be very interesting.

Furthermore, exploring the genome-wide effects of E2F-1 using DNA microarrays has revealed a large number of genes that are regulated by E2F-1 in diverse cellular processes, including replication, apoptosis, checkpoint control and DNA repair (Ren *et al.*, 2002; Ma *et al.*, 2002; Polager *et al.*, 2002; Stanelle *et al.*, 2002 and Table1.1). Taken together with the results presented in this thesis, these studies suggest a role for E2F-1 in regulating DNA damage checkpoint responses, DNA replication and possibly facilitating efficient DNA repair. Studies aimed at determining whether P-S364 E2F-1 co-localises to damage induced nuclear foci with activated Chk2 have been hampered by the fact that the P-S364 antibody and the P-Thr68 Chk2 antibody are both rabbit polyclonal in origin. More studies are required to determine whether P-S364

E2F-1 co-localises with other proteins involved in checkpoint control such as BRCA1 and the Mre11 complex.

## 8.8. General discussion

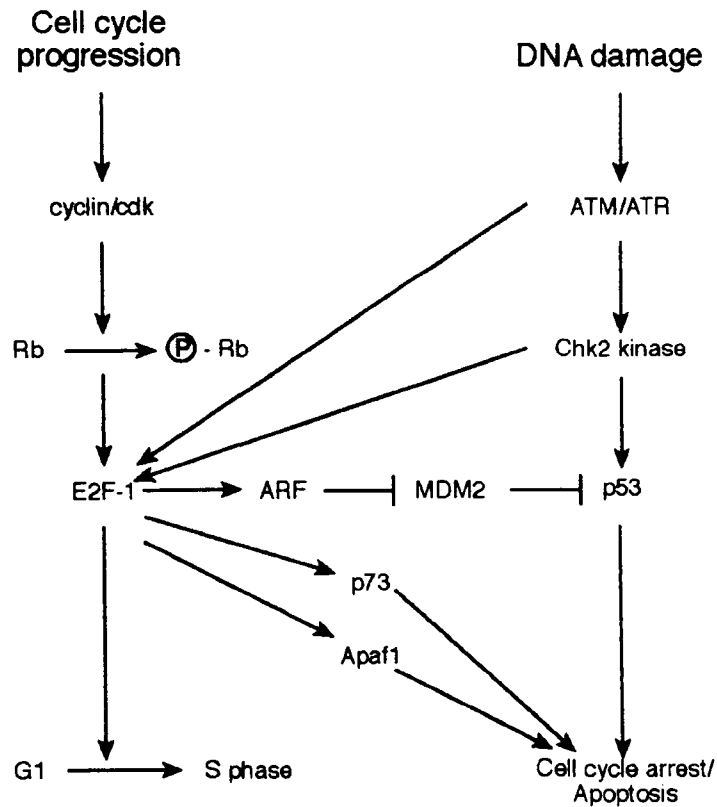
The results presented in this thesis establish E2F-1 as a physiological target for Chk2 kinase in response to DNA damage. In response to etoposide treatment, Chk2 phosphorylates E2F-1 on Ser364 leading to an increase in the stability of E2F-1, and in turn the increased abundance of E2F-1 leads to cell cycle arrest and apoptosis through a transcription based mechanism. The observation that E2F-1 phosphorylation is induced by DNA damage defines E2F-1 as a likely effector protein in the cellular response to DNA damage.

E2F-1 can regulate cellular growth in both a positive and negative manner. For example, while E2F-1 can stimulate cell cycle progression by controlling the expression of genes required for DNA synthesis and entry into S-phase, it can also play a role in cell cycle arrest and apoptosis (Trimarchi and Lees, 2001). A number of reports have illustrated this duality in E2F-1 function. For example, E2F-1 can function as an oncogene *in vitro* and *in vivo* (Johnson *et al.*, 1993; Johnson *et al.*, 1994; Lukas *et al.*, 1996; Yamasaki *et al.*, 1998), and cells lacking E2F-1 exhibit cell cycle defects (Degregori *et al.*, 1997). In contrast, several reports have described the apoptotic activity of E2F-1 (Qin *et al.*, 1994; Shan *et al.*, 1994; Wu *et al.*, 1994), which may explain the increased tumour incidence and reduced levels of apoptosis observed in *E2F-1*<sup>-/-</sup> mice (Field *et al.*, 1996; Yamasaki *et al.*, 1996). The pathway described here for

Chk2 in regulating E2F-1 abundance and apoptosis may be involved in controlling the tumour suppressor-like activity previously ascribed to E2F-1.

The results argue that Chk2 induces E2F-1 and in turn provides a signal for cell cycle arrest and apoptosis. In this respect, the damage induction of E2F-1 resembles the p53 response, which similarly results in cell cycle arrest and apoptosis. The physiological significance of the apoptosis signalled by p53 and E2F-1 may relate to the severity of damage where, in situations of extreme damage, it is beneficial for the cell to activate apoptosis rather than attempt DNA repair. Alternatively, it makes sense that the cell has more than one mechanism through which it can cause cell cycle arrest or apoptosis. Thus E2F-1 may provide a safeguard mechanism in the event that p53 function is disabled. Whilst it has already been reported that E2F-1 and p53 can act synergistically to induce apoptosis (Wu and Levine, 1994), the molecular mechanisms involved are not yet clear. Future studies aimed at identifying genes such as *Apaf-1* that are regulated by both E2F-1 and p53 will provide a better mechanistic understanding of the integration of these two checkpoint pathways.

Taken together with another study (Lin *et al.*, 2001) the results presented here establish that both Chk2 and ATM signal DNA damage to E2F-1. Thus, in normally growing cells, E2F-1 along with E2F-2 and E2F-3 is involved in the control of the transition from G1 into S-phase. However, in response to DNA damage, genes such as *ARF*, *p73* and *Apaf-1* are induced through damage-responsive signalling to E2F-1 which, in turn and perhaps in concert with p53 target genes, result in cell cycle arrest or apoptosis (Figure 8.2).



**Figure 8.2 Summary of the E2F-1 pathway and the p53 response to DNA damage**

A summary of the pRb/E2F pathway (left) and p53 response to DNA damage (right), together with ATM/ATR and Chk2 kinases involved in DNA damage signalling to p53. Targets that may result in E2F-dependent apoptosis, such as ARF, p73, and Apaf-1 are indicated. The arrow connecting Chk2 directly with E2F-1 is derived from the conclusions in this thesis and emphasises the interplay between the DNA damage signalling pathway and the pRb/E2F pathway. The connection between ATM/ATR and E2F-1 is based on the study by Lin *et al.*, 2001.

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